The role of free glucose as carbon metabolite during intracellular growth of *Listeria monocytogenes*

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i. ABSTRACT

An adequate multiplication rate *in vivo* is crucial for an infectious agent to cause clinical disease and achieve its transmission to new hosts. Despite their key importance, the nutritional and metabolic determinants of pathogen proliferation within the host remain a neglected area of research in infection biology. In this MSc thesis I have investigated the carbon metabolites used by the bacterial intracellular pathogen *Listeria monocytogenes* to grow within host cells.

Previous work in the Vazquez-Boland laboratory demonstrated that, via a specific permease named Hpt, *L. monocytogenes* steals hexose phosphates from the host cell to fuel its rapid intracellular growth. Albeit more slowly, mutants lacking this permease are still able to replicate intracellularly, indicating that *Listeria* uses other carbon substrates from the host cell. Evidence suggested that free glucose could be this additional carbon substrate. To test this hypothesis, I sought to obtain a glucose utilisation-deficient mutant by disabling the main transport systems involved in glucose uptake by *L. monocytogenes*. A double mutant was constructed which lacked the central component of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) Hpr (PtsH) necessary for PTS-dependent sugar transport, plus the main non-PTS specific glucose transporter GlcU1. The double *ptsH*/*glucU1* knock-out mutant was virtually unable to grow on glucose *in vitro* and to proliferate within HeLa epithelial cells in conditions in which free glucose is in excess, indicating that glucose is a main intracellular carbon source for *L. monocytogenes*.

I also sought to provide evidence for this conclusion from the host cell side. Using siRNA knockdown assays, HeLa cells were depleted of hexokinase, the enzyme that converts all the incoming free glucose into glucose-6-phosphate. A Δ*hpt* mutant unable to use hexose phosphates showed wild-type growth in the hexokinase-depleted host cells, further indicating that free glucose, before conversion into glucose-6-phosphate and entering glycolysis, is a major carbon substrate for intracellular *Listeria*.

ii. ACKNOWLEDGEMENTS

Many thanks first and for most to my supervisor Professor Jose Antonio Vázquez-Boland for all his help and support.

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Lastly and most importantly I would like to thank all my colleagues from Vazquez-Boland group at University of Edinburgh that supported and encouraged me everyday during the project.

A mi familia por todo su apoyo.

iv. AUTHOR'S DECLARATION

I declare that the work included in this dissertation was carried out in accordance with the Regulations of the University of Edinburgh. The work is original, except where indicated by special reference in the text, and no part of the thesis has been submitted for any other academic award.

Any views expressed in the thesis are those of the author.

The thesis has not been presented to any other University for examination either in the United Kingdom or overseas.

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Bovine serum albumin	BSA
Brain Heart Infusión media	BHI
Calcium chloride	Cl_2Ca
Chloranphenicol	Cm
Colony form units	CFU
Degrees	°C
Desoxyribonucleic acid	DNA
Dulbeco's modified Eagle's medium	DMEM
Escherichia coli	*E. coli*
Erythromicin	Em
Foetal serum bovine	FBS
Glucose-1-phosphate	G1P
Glucose-6-phosphate	G6P
Glucose-6-phosphate deshydrogenase	G6PDH
Glucokinase	GK
Grames	g
Hexokinase	HK
Hexose phosphate	HP
Hour	h
Improved minimal media	IMM
Internalin A	Inl A
Internalin B	Inl B
Internalin C	Inl C

Listeria monocytogenes	*L. monocytogenes*
Listeriolysin O	LLO
Liter	l
Luria-Bertani media	LB
Microfarad	µF
Micrograms	µg
Microliter	µl
Micromolar	µM
Miligrames	mg
Mililiter	ml
Milimeters	mm
Milimolar	mM
Minutes	min
Molar	M
Nanograms	ng
Ohms	Ω
Optical density at 600nm	OD_{600}
Phosphate-buffered saline	PBS
Phospholipase A	PlcA
Phospholipase B	PlcB
Polymerase chain reaction	PCR
Revolutions per minute	Rpm
Seconds	S
Standard Error	SE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Volt	V
Water	H_20

Introduction

1.1 *Listeria*

The genus *Listeria* contains eight species, of which only *L. monocytogenes* and *L. ivanovii* are able to cause disease, the rest of them (*L. seeligeri, L. innocua, L. welshimeri, L. grayi, L. marthii and L. weihenstephanensis*) are not pathogenic (Graves *et al.,* 2010; Halter *et al.*, 2013). From the pathogenic species of *Listeria, L.monocytogenes* is the most important infection agent in humans being also able to infect animals. Only a few human infection cases have been reported to be caused by *L. ivanovii* that mostly infects ruminants (Guillet *et al,*. 2010)

Listeria are Gram-positive rods with low G+C content, unable to form capsule or spores, and motile at temperatures between 10 to 25ºC (Grundling *et al.*, 2004). *Listeria* are facultative anaerobic bacteria capable to grow at temperatures between 4 to 42°C, in a broad range of pHs (4.3 – 9.6) and salt concentrations (< 10%) (Collins *et al.*, 1991, Rocourt, 1999). Decomposing plant matter is the bacteria natural habit where they live like saprophytes, but they can be isolated from different environments such as water, soil, animal and human feces and food.

1.2 *Listeria* pathogenesis and listeriosis

The infectious disease caused by *Listeria* is known as listeriosis. Although *Listeria* can infect healthy adults, it has been described as an opportunistic pathogen primarily affecting pregnant women, neonates and elderly people. The disease can vary from a non-invasive form, generally an

asymptomatic infection restricted to the gastrointestinal tract, to an invasive form causing sepsis, and eventually meningoencephalitis and abortion (Vazquez-Boland *et al.*, 2001a).

First cases of human listeriosis were reported in 1929 in Denmark (Nyflet, 1929) but the disease at that time was rare. It was not until the beginning of the 1980s when listeria was established as an important food-borne pathogen, causing epidemic outbreaks in human in North America and Europe (Ryser, 1999; Goulet *et al.*, 2001; Roberts & Wiedmann, 2003; de Valk *et al.*, 2005; Goulet *et al.*, 2008).

Listeria infection is often associated with contaminated food ingestion (Farber & Peterkin, 1991). Bacteria pass through the stomach to penetrate in the host intestinal epithelium (Figure 1) (Nikitas *et al.*, 2011). From there, *Listeria* raises the lymph nods and gets to the primary target organs spleen and liver via lymphohaematogenous dissemination (Marco *et al.*, 1992; Pron *et al.*, 1998; Bou Ghanem *et al*, 2012). If the infection is not controlled at this point, bacteria can invade secondary target organs such as brain or placenta. In those cases there is a high risk of meningoencephalitis, abortion or neonatal septicemia (Vazquez-Boland *et al.*, 2001a).

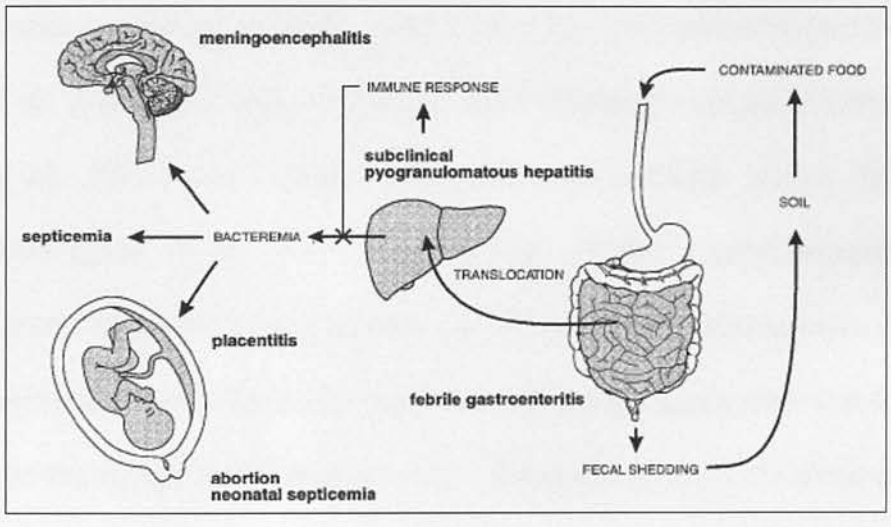

Figure 1. Representation of *Listeria* infection pathophysiology (Vazquéz-Boland *et al.*, 2001).

1.3 Intracellular cycle

Listeria can invade, proliferate and spread in phagocytic cells and many mammalian cell types. Non-phagocytic cells are invaded via specific ligand-receptor interactions (Kuhn and Goebel, 2000).

The intracellular cycle starts with the adhesion and internalization of the bacteria into the host cell. *L. monocytogenes* interacts with a number of different eukaryotic receptors including the transmembrane glycoprotein E-cadherin (Mengaud *et al.,* 1996), the C1q complement factor receptor (Braun *et al.,* 2000), the Met receptor for the Hepatocyte Growth Factor (HGF) (Shen *et al.,* 2000) and components of the extracellular matrix (ECM) (Alvarez-Dominguez *et al.*, 1997; Gilot *et al.*, 1999). Ligand-receptor interactions induce bacteria internalization by a zipper-type mechanism in which each bacterium gradually sinks into host cell pseudopods until it is finally engulfed into the cytoplasm (Figure 2a, 2b) (Schubert *et al.,* 2002;Shen *et al.*, 2010).

Inside the cell, *L. monocytogenes* is initially enclosed in a phagocytic vacuole and avoids lysosome formation by disrupting the phagosome membrane. (Figure 2c) (Alvarez-Dominguez *et al.*, 1997; Gaillard *et al.*, 1987). Thus, *Listeria* is free in the cytosol and starts the replication phase (Vazquéz-Boland *et al.,* 2001). At the same time, *L .monocytogenes* induce actin polymerization and the bacteria became surrounded by a cloud of actin filaments. Once the daughter bacteria are detached from the mother, actin filaments rearrange in one pole of the bacterium forming the characteristic *Listeria* tails (Figure 2d). Two populations of cross-linked actin filaments

conform each tail that propels *Listeria* in to the cytosol following a random circulation known as 'rocket movement'. This movement allows the bacteria to reach the periphery of the cell and eventually being in contact with the plasma membrane to create pseudopod-like structures that protrudes into neighboring cells. These protuberances with a bacterium in the top are engulfed by phagocytosis, leading to the formation of a double membrane phagosome in the adjacent cell (Figure 2e). Then *L. monocytogenes* intracellular cycle starts again with the bacteria escaping from the vacuole, replicating and spreading (Vazquez-Boland *et al.,* 2001; Hamon *et al.,* 2006).

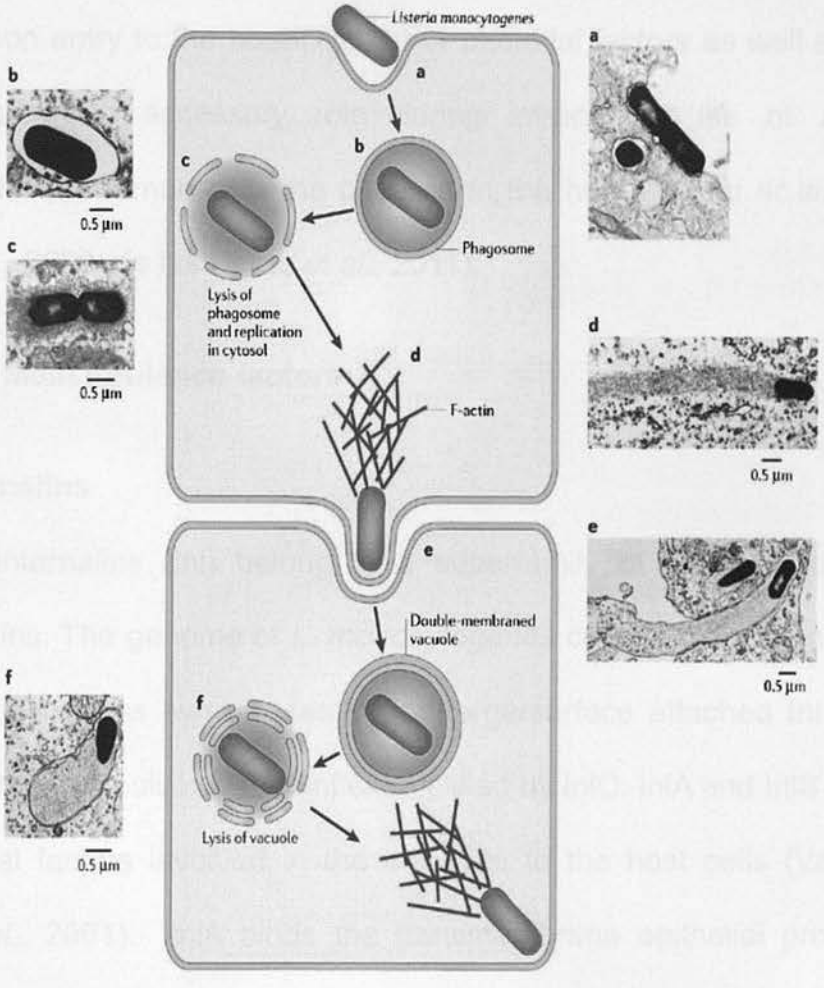

Figure 2. Intracellular life cycle of *L. monocytogenes*. (a) *L. monocytogenes* induces its entry into a non-professional phagocyte; (b) bacteria are internalized in a phagocytic vacuole; (c) the membrane of the vacuole is disrupted by the secretion of two phospholipases, PlcA and PlcB, and the pore-forming toxin listeriolysin O; (d) Bacteria are released into the cytoplasm where they multiply and start to polymerize actin, as observed by the presence of the characteristic actin tails; (e) actin polymerization allows bacteria to move inside the cytosol and eventually pass into a neighbouring cell by forming protrusions in the plasma membrane; (f) upon entry into the neighbouring cell, bacteria are present in a double-membraned vacuole, from which they can escape to perpetuate the cycle. (Hamon *et al.*, 2006).

1.4 *L. monocytogenes* virulence

All the steps of the intracellular life cycle of *L. monocytogenes* are mainly mediated by only few key virulence factors that are under the control of the main virulence regulator PrfA (positive regulatory factor A), which is activated upon entry to the host cell. Other bacterial factors as well as gene regulators play an accessory role during intracellular life of *Listeria,* enhancing the performance of the bacteria in the host (Scortti *et al.,* 2007; Freitag *et al.,* 2009; de las Heras *et al.*, 2011).

1.4.1 Main virulence factors

Entry: Internalins

The internalins (Inl) belong to a superfamily of group leucine-rich repeat proteins. The genome of *L. monocytogenes* contains more than 20 *inl* genes which encodes two classes of Inl: large/surface attached Inl as the InlA and InlB; and small/excreted Inl exemplified by InlC. InlA and InlB are the major listerial factors involved in the entrance to the host cells (Vazquez-Boland *et al.,* 2001). InlA binds the transmembrane epithelial protein E-cadherin mediating the bacteria gastrointestinal invasion (Mengaud *et al.,* 1996; Schubert *et al.,* 2002). InlB binds the c-Met receptor and the Hepatocyte Growth Factor (HGF) promoting invasion in a broad range of mammalian cell types (Shen *et al.*, 2010). It has been recently shown that InlB can work synergistically with InlA not only to promote feto-placental

infection but also accelerating the intestinal invasion (Pentecost *et al.,* 2010).

Phagosome disruption: Listeriolysin O and Phospholipases

Listeriolyisin O, LLO, was the first virulence determinant to be identified and sequenced in *L. monocytogenes* (Jenkins *et al.,* 1964). This protein is a streptolysinO-related cytolysin belonging to the family of cholesterol-dependent pore-forming toxins (Geoffroy *et al.,* 1987). LLO works in a low pH (similar to the one within the phagosome) creating pores in the phagosome membrane (Gedde *et al.,* 2000). LLO has a PEST-like sequence (P, Pro; E, Glu; S, Ser, T, Thr) present in the N-terminus for a fast degradation in the host cell cytosol; thus the bacterium avoids any damage that could compromise host cell viability (Decatur and Portnoy, 2000). The pores caused by LLO facilitate the action of the listerial phospholipases leading to total disruption of the phagosome membrane. It has been recently published that LLO is also involved in *L. monocytogenes* internalization (Vadia *et al.,* 2011).

L. monocytogenes secretes two phospholipases C with different substrate specificity: PlcA and PlcB. PlcA is a phosphatidylinositol-specific phospholipase while PlcB is a lecithinase with a broad range of substrates including phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine (Vazquéz-Boland *et al.,* 2001). PlcA is an accessory virulence factor that helps LLO and PlcB for the bacteria escape from the primary phagocytic vacuole (Smith *et al.,* 1995; Alberti-Segui *et al.,* 2007). PlcB also has a role dissolving the primary and secondary vacuoles independently of LLO activity (Gründling *et al.*, 2003).

Movement and cell-to-cell spread: Actin nucleator protein

The actin nucleate protein, ActA, is a surface-associated protein that mediates the actin-based motility of the bacteria promoting cell-to-cell spread. ActA activates the actin polymerizing protein complex Arp2/3 of the host cell and the recruiting G-actin (Kocks *et al.,* 1992; Cossart, 2000). ActA takes also part in the host cell invasion directing an internalization pathway specific for epithelial cells, remodeling the cells microvilli into pseudopods that mediates bacterial uptake (Suarez *et al.,* 2001). A recent publication shown that ActA also promote bacterial aggregation in the host intestine, which is associated with a longer bacterial persistence and shedding through the feces, therefore contributing to listerial transmission (Travier *et al.,* 2013).

Replication: Hexoses phosphate transporter

The hexoses phosphate transporter, Hpt, is a permease homolog to the mammalian glucose-6-phosphate translocases (G6PT) only present in *Listeria* pathogenic species. Hpt belongs to the organophosphate:inoraganic phosphate antiporter family of transporters with homologous in other pathogens as *Escherichia coli*, *Salmonella enterica, Shigella flexneri* and *Staphylococcus aureus* (Chico-Calero *et al.,* 2002) among others. Hpt mediates the uptake of hexoses phosphates from the host cell cytosol through a P(i)-linked antiport carrier to boost an efficient *listerial* intracellular replication. (Chico-Calero *et al.,* 2002).

1.4.2 Regulation

PrfA

PrfA is a member of the (Crp)/fumarate nitrate reductase regulator (Fnr) family, specific to *Listeria* pathogenic species. The core regulon of PrfA contains 10 genes, 6 of them clustered in a pathogenicity island (LIPI-1) (Milohanic *et al.,* 2003; De las Heras *et al.,* 2011) (Figure 3). PrfA acts as a switch that turns on-off the expression of the virulence genes, optimizing the fitness of the bacteria when growing either in the infected host or as a saprophyte in the environment (de las Heras *et al.,* 2011). The exact mechanism of induction is unknown but there is strong evidence that it is mediated by allosteric activation of the protein upon binding a co-factor (de las Heras *et al.,* 2011; Deshayes *et al.,* 2012).

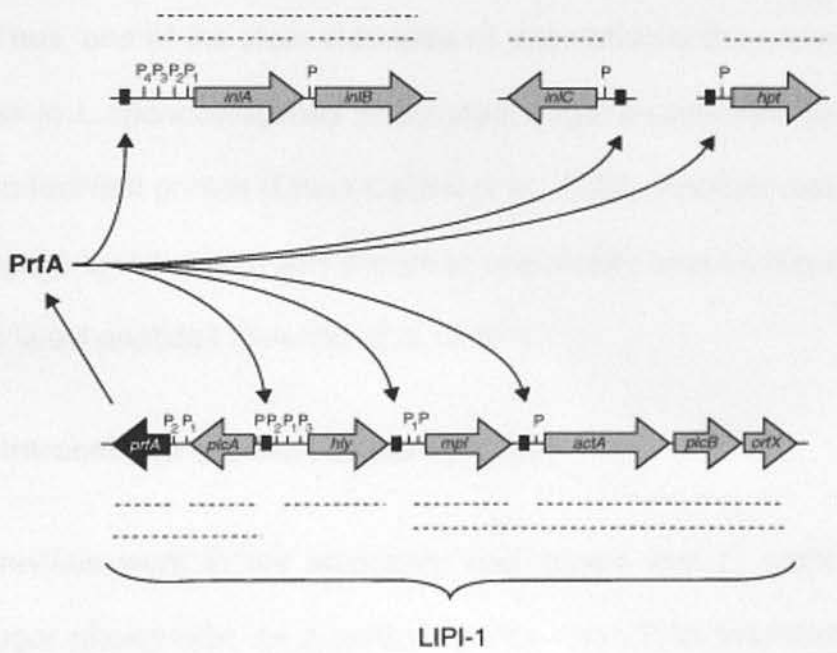

Figure 3. The PrfA regulon. The PrfA regulon contains 10 genes, 6 of them clustered in the *Listeria* pathogenicity island (LIPI-1): *hly* , *actA, plcA, plcB, mpl* and *prfA*; plus three additional chromosomal loci: the *inlAB* operon and the *inlC* and *hpt* monocistrons. Genes pointing to the right are on the positive strand. PrfA boxes are indicated by black squares, known promoters and transcripts are indicated by 'P' and dotted lines respectively (modified from De las Heras *et al.*, 2011).

1.5 *L. monocytogenes* intracellular replication

Within the host cells *Listeria* replicates in the cytosol with a longer doubling time compared with growth in rich medium (≈ 60 min in J774 cells and ≈ 20 min in rich medium), nevertheless the cytosol seems to provide *Listeria* with all the necessary nutrients for growth. Although the cytosol is considered, in nutritional terms, a rich environment not all intracellular bacteria are able to replicate in the cytoplasm as demonstrated by Goetz and coauthors (2001). They shown that bacteria non-adapted to live in the cytosol as the intravacuolar *Yersinia enterocolitica* and *S. typhimurium* were unable to replicate efficiently in the cytoplasm when directly placed there by microinjection (Goetz *et al.*, 2001). These results suggest that cytosolic bacteria require specific adaptations to reach an efficient replication in this niche. Thus, one of the clear examples of adaptation is the presence of Hpt permease in *L. monocytogenes* which steal sugar phosphates from the host cytosol to fuel fast growth (Chico-Calero *et al.,* 2002). Another example is the lipoate ligase LplA1, which was shown to specifically adquire lipoate from the cytosolic lipoyl peptides (Keenay *et al.,* 2007).

1.5.1 Intracellular carbon related nutrition

Previous work in our laboratory had shown that *L. monocytogenes* utilize sugar phosphates as a carbon source upon PrfA activation (Ripio *et al.,* 1997). Moreover, Chico-Calero *et al.* (2002) published that Hpt is required for efficient proliferation in host cell and essential for *L. monocytogenes* full virulence in mice. However, mutants lacking the Hpt permease, although

impaired in growth (at least twice the doubling time of the wild-type strain) can still proliferate in the host cytosol despite they are unable to use sugar phosphates (Figure 4) (Chico-Calero *et al.,* 2002). These data suggests that *L. monocytogenes* uses other cytosolic nutrients as carbon sources while utilizing hexoses-phosphate as an additional source of carbon for a rapid intracellular growth.

Recent studies carried out in Vazquez-Boland's laboratory using Hpt^- and Hpt^+ *Listeria* shown that the intracellular proliferation defect of Hpt^- bacteria was only evident when the host cells were kept at or below physiological concentrations of glucose (≤ 5mM) but not when glucose was added at higher concentration to the culture media (25mM) (Figure 5). They also observed that when the host cell was deprived of glucose *L. monocytogenes* did not proliferate whereas other intracellular bacteria as *S. enterica* and *S. aureus* were not affected in growth. Therefore, taking together these data it was suggested that *Listeria* depend on a glucose-derived carbon source for intracellular growth and that the non-sugar phosphate based growth (Hpt^-) is supported by free glucose or a metabolite directly derived from glucose.

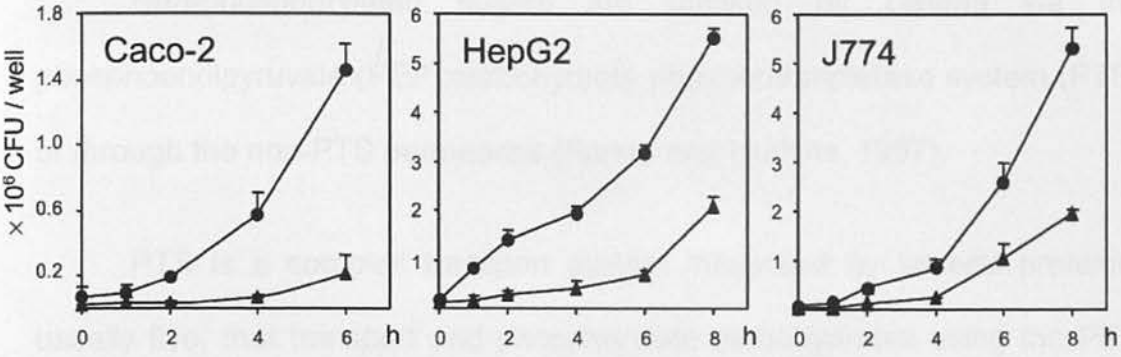

Figure 4. Effect of the Hpt mutation on the intracellular replication of *L. monocytogenes* in mammalian cells. Human colon epithelial cells (Caco-2), human liver hepatocellular carcinoma cells (HepG2) and mouse monocyte macrophages (J774) were infected with Hpt$^+$ and Hpt$^-$. Results are the mean of at least three independent experiments (each performed in duplicate) ± SE. wt/ Hpt$^+$ (●) and Δ*hpt*/ Hpt$^-$ (▲). (Chico-Calero *et al.*, 2002)

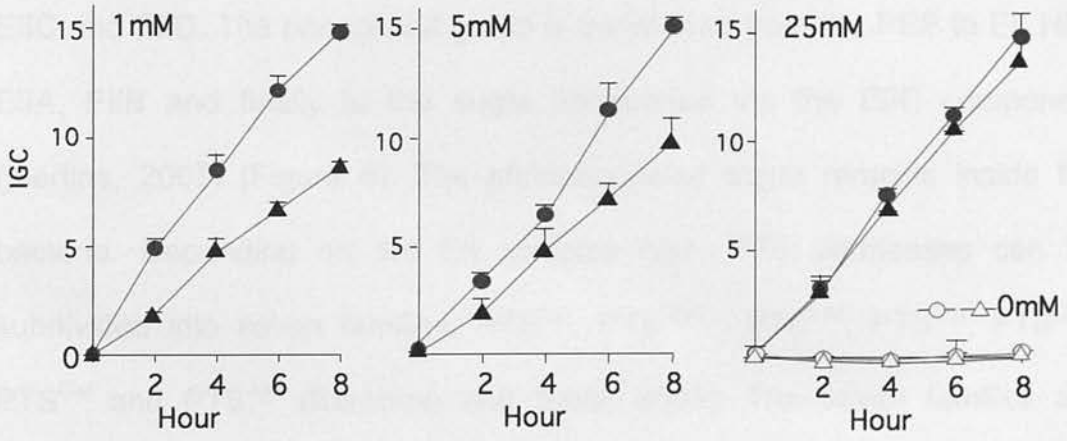

Figure 5.Effect of different intracellular glucose concentrations on the *L. monocytogenes* Hpt$^-$ and Hpt$^+$ replication in rat hepatoma FTO2B cells. Results are the mean of at least three independent experiments (each performed in duplicate) ± SE
wt/ Hpt$^+$ (● / ○) and Δ*hpt*/ Hpt$^-$ (▲ /△). IMM+ 5mM glucose are physiological conditions (Lecharme-Lora, PhD thesis, 2007).

1.5.2 Transport of glucose in *L. monocytogenes*

Non-phosphorylated sugars are uptaked by *Listeria* via the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) or through the non-PTS permeases (Parker and Hutkins, 1997).

PTS is a complex transport system integrated by several proteins, usually five, that transport and phosphorylate carbohydrates using the PEP as a phosphoryl donor (Kundig *et al.*, 1964). In *Listeria* the system is redundant with 86 *pts* genes encoding 29 complete PTSs (Barabote and Saier, 2005; Stoll and Goebel 2010). The system consists basically of two cytoplasmic sugar un-specific proteins: HPr and enzyme I (EI), and the membrane-associated enzyme II (EII) complexes specific for one or few sugars. The EII complex consist of 3 to 4 proteins or domains: EIIA, EIIB, EIIC and EIID. The phosphoryl group is transferred from the PEP to EI, HPr, EIIA, EIIB and finally to the sugar transported via the EIIC component (Mertins, 2007) (Figure 6). The phosphorylated sugar remains inside the bacteria. Depending on the EII complex type, PTS permeases can be subdivided into seven families: PTS^{Glc}, PTS^{Man} , PTS^{Lac}, PTS^{Fru}, PTS^{Glut}, PTS^{Gat} and PTS^{Asc} (Barabote and Saier, 2005). The seven families are present in *L. monocytogenes* where PTS^{Man-2}, PTS^{Man-3} and PTS^{Glc-1} are the main permeases involved in the glucose up-take, although with different efficiency (Stoll and Goebel, 2010).

L. monocytogenes also encodes a low-affinity glucose transport system in which the internalization of glucose is probably driven though

permeases by the proton motive force (PMF) (Christensen and Hutkins, 1994). Recent work from Aké *et at* (2011) described three genes encoding three low-affinity non-PTS glucose transporters GlcU1, GlcU2 and GlcU3, homologues to those of *S. xylosus* and *Lactococcus lactis*. Their studies revealed that the low affinity glucose transporters do not contribute significantly to the glucose uptake. The function of these transporters was described as accessory and no effect in the bacteria growth has been observed when each gene was deleted.

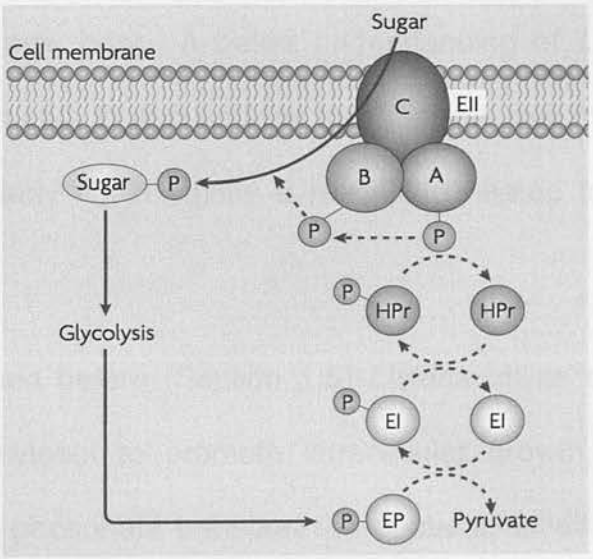

Figure 6. Schematic representation of the PTS system. The PTS is composed of five distinct proteins: the enzyme II complex (EII) that consists of three proteins: EIIA, EIIB, EIIC; protein (HPr); and enzyme I (EI) (Nancy et al., 2009).

1.6 Justification and objectives

Infection due to *L. monocytogenes,* although has lower incidence compared with another foodborne diseases such as salmonellosis, has a high overall fatality rate of 20-30%, reaching 70% in untreated neurologic cases and 50% in infected newborns. The intracellular location of *listeria* on one side hide the pathogen from the immune system and, on the other side make difficult to treat the infection because only few antibiotics are able to reach the cell cytosol at high concentration.

An important step in the infection process is the adequate replication of the parasite to increase the bacterial load and eventually produce disease and spread to a new host. A better understanding of *L. monocytogenes* nutritional requirements *in vivo* and the mechanisms that the bacterium uses to grow intracellularly could signify a revolution related to new treatments against listeriosis.

As mentioned before (Section 1.5) *Listeria* utilize sugar phosphates from the host's cytosol to promote intracellular growth, however in the absence of sugar phosphate transport the bacteria, albeit slow, are able to growth intracellularly. Preliminary work from Vazquez-Boland's group indicates that *Listeria* utilizes free glucose from the host cytosol as a source of carbon to enable growth (see Section 1.5.1). The aim of this thesis is to further explore the utilization of cytosolic free glucose as a carbon source for listerial growth.

More specifically, the objectives of this Master by Research thesis are:

1. To obtain more direct evidence that glucose -or an early intermediate of the glycolitic pathway- is the main carbon source for *L. monocytogenes* inside the host cell.

2. To assess the role of the PTS system and the non-PTS glucose transporters during listerial intracellular replication.

2. Results

Role of glucose transporters during intracellular *listerial* replication

L. monocytogenes uptakes glucose and another non-phosphorylated sugars mainly via the PTS system (Stoll and Goebel, 2010). In *Listeria* this system is redundant to the extent that 3.2% of all *L. monocytogenes* genes encode PTS proteins (Barabote and Saier, 2005). Recently, Stoll and Goebel (2010) had shown that in *L. monocytogenes* PTS^{Man-2}, PTS^{Man-3} and PTS^{Glc-1} are the main PTS systems involved in the uptake of glucose. *L. monocytogenes* mutants lacking those transporters are severely impaired during *in vitro* growth under low glucose concentrations (< 2mM). However Stoll and Goebel (2010) did not observed any effect during intracellular replication in the Δpts^{Man}, pts^{Glc-1} mutant (defective in all four PTS^{Man} and PTS^{Glc-1} permeases).

Given the redundancy of these transporters in *L. monocytogenes* and the lack of precise knowledge about the regulation of the different PTS transporters under different conditions we decided to use a mutant in the common PTS proteins to analyze the contribution of the glucose to the *listerial* intracytosolic growth. A *L. monocytogenes* Δ*ptsH* mutant lacking functional phosphoryl donor protein HPr was already available in our laboratory. Mutants in the general PTS proteins had been reported to have an impaired growth *in vitro* when glucose is the only carbon source present in the culture media, however they never been tested in *in vivo* conditions (Mertins *et al,* 2007, Stoll and Goebel, 2010; Aké *et al.,* 2011).

Recent publication from Aké and collaborators (2011) suggested that the non-PTS glucose transporters GlcU could be responsible for the glucose uptake in absence of a functional PTS system, although single mutants defective in the three GlcU transporters did not show any effect in the bacteria *in vitro* growth (Aké *et al.,* 2011).

In order to obtain bacteria totally impaired to grow in glucose, a disabled mutant in GlcU1 (main GlcU transporter) was generated by plasmid insertion on *L. monocytogenes* Δ*ptsH* (already lacking PTS transport).

2..1 Construction and characterization of *ptsH, glcU1* mutants in *L. monocytogenes*

All the bacterial strains used in this project are summarized in Table 1, section 5.1.2 Materials and Methods.

L. monocytogenes Δ*ptsH* in-frame deletion mutant was obtained by double homologous recombination and it was already available in Vazquez-Boland strain collection. Single insertion mutant *glcU1*::pAUL-A and double mutant Δ*ptsH,glcU1*::pAUL-A were generated by disruption of *glcU1* gene through the insertion of pAUL-A plasmid (Material and Methods, Section 5.2.3).

We complemented Δ*ptsH* using a modification of the site-specific shuttle integration vector pPL2 (Materials and Methods, section 5.2.4). The phenotypic characterization of Δ*ptsH*(pPL2Pδ*ptsH*) didn't show any difference with the parental strain (Section 2.1.2, Tables 6 to 10) and its

intracellular replication did not differ from the native wild type. That showed the function of the *ptsH* gene has been restored, probing that the decrease in the proliferation levels of Δ*ptsH* strains was caused exclusively by the lack of *ptsH* gene. We found no need to complement the gene *glcU1* because we couldn't find significant differences between intracellular *glcU1*::pAUL-A and the parental strain.

A phenotypic characterization of each strain was performed to exclude any defect in the activity of PrfA that may influence the virulence of the *L. monocytogenes* mutant strains. To this end, we analyzed the phenotype using two natural reporters of the PrfA funtionability: PlcB and Hpt. The analysis of Hpt allows us also to rule out any defect in the transport of HPs that can impair the bacteria growth inside the host cell.

The activity of the PlcB was tested by restreaking the bacteria in lecithin plates with and without 0.5% activated charcoal supplementation (Material and Methods, Section 5.4.2). *L. monocytogenes* hydrolyze the lecithin present in the medium through the action of the PrfA-dependent PlcB phospholipase developing a visible opacity halo around the bacteria streak. During *in vitro listerial* growth PrfA-dependent genes are weakly expressed and therefore no precipitation halo is observed. However, when activated charcoal is present, the PrfA system is upregulated (Ermolaeva *et al.,* 2004) and the halo is observed around the strains. All the mutant strains behaved similar to the parental strain, showing a halo only when the charcoal was present in the media and the PrfA became active (Figure 7).

The activity of the Hpt was tested using a sugar-phosphates fermentation assay (Figure 8). This test is based on the pH meter capacity of the phenol red broth that changes its colour from red to yellow when the bacteria utilize the sugar generating acids that decreases the pH. *hpt, as* another PrfA-dependent virulence factor, is only expressed when PrfA is active and therefore the test was carried out using media with and without active charcoal. The results were the same for all the tested strains (Table 1, section 5.1.2 Materials and Methods): while phenol-red test was negative (red) because Hpt was not able to transport the HP sugar, The phenol-red charcoal test was positive (yellow) showing that when the PrfA system gets active, Hpt is functional for all the strains (Figure 8).

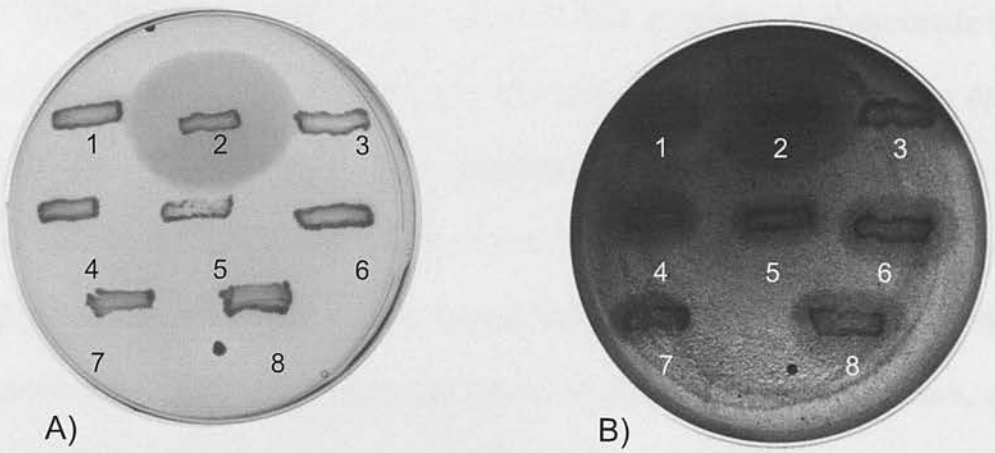

Figure 7. PrfA phenotype based on lecithinase activity. A) Lecithin plate B) Lecithin + charcoal plate. Halos show the lecithin hydrolyzation and therefore corresponds to the activity of PrfA. A Halo around bacteria is observed for $PrfA^{WT}$ phenothype; Bigger halo is observed when PrfA is over expressed (PrfA* phenotype) and no halo is observed for PrfA- phenotype. Experiment control phenotype: $PrfA^{WT}$ (1), PrfA* (2), PrfA- (3). Strains to test: *ΔptsH*(pPL2-Pδ*ptsH*) (4), *ΔptsH* (5), WT (6) *ΔptsH,glcU1*:: pAUL-A(7) and *glcU1*:: pAUL-A (8).

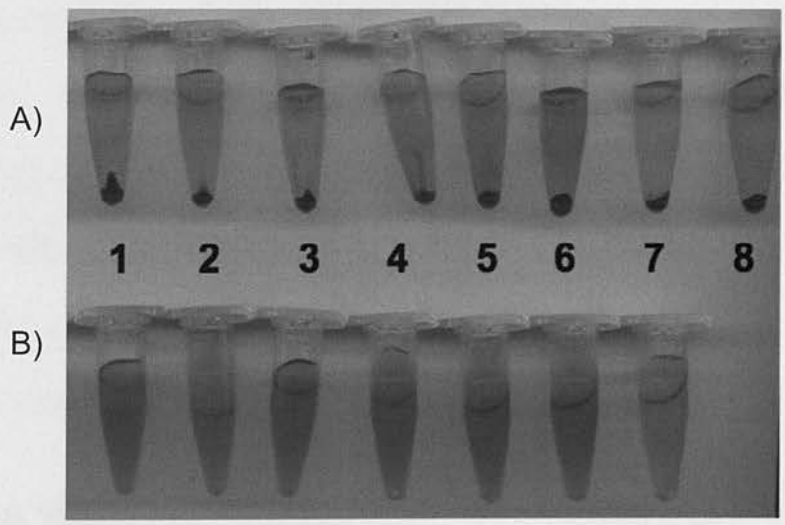

Figure 8. Hpt phenotype based on fermentation capacity. A) Phenol red + charcoal B) Phenol red. A change in the color from red to yellow is observed for strains with a functional hpt. Strains to test: *ΔptsH*(pPL2-Pδ*ptsH*) (1), *ΔptsH* (2), WT (3), *ΔptsH,glcU1*::pAUL-A (4) and *glcU1*:: pAUL-A (5). Experiment control phenotype: $PrfA^{WT}Hpt^{-}$ (6), $PrfA^{*}Hpt^{WT}$ (7), $PrfA^{WT}Hpt^{WT}$(8).

2.1.2 Effect of single and double mutation on *in vitro* growth

Six different broths were used in this phenotypic characterization, growing every strain during 24h and 48h and taking measures of the optical density at 600 nm (OD_{600}) every 30 minutes. Three preliminary experiments were performed using laboratory routine media Brain Heart Infusión media (BHI), Luria-Bertani (LB) and LB supplemented with 20mM glucose. BHI is a sugar-reach media use to grow *Listeria* in an everyday laboratory work, while LB is a media poor in glucose where *L. monocytogenes* struggles to grow. By doing this first experiment, we wanted to compare the growth of the different strains (trying to find differences from the wild type) by the addition of glucose to the poor glucose media LB.

In BHI (Figure 9A), the single mutant for the non-PTS glucose transporter GlcU1 (*glcU1*::pAUL-A) grew as well as did the parental strain EGD. Only the single mutant in the PTS system (Δ*ptsH*) and the double mutant for the PTS system and the non-PTS transporter GlcU1 (Δ*ptsH,glcU1*::pAUL-A) had a considerable delay in their growth. Δ*ptsH* reached wild type growth levels after 20h (OD_{600} of 1.2 -1.4), Δ*ptsH,glcU1*::pAUL-A showed a slight growth reduction even after 24h of incubation (OD_{600} of 1).

In LB (Figure 9B), were the growth is lower than in BHI, the growth of the double and single mutant strains Δ*ptsH* and Δ*ptsH,glcU1*::pAUL-A was specially affected. These two strains had a long log phase and their growth

was delayed 15h compared with the wild type strain.

When glucose was added to the LB broth (Figure 9C), the growth of Δ*ptsH* and Δ*ptsH,glcU1*::pAUL-A was highly delayed (5h and 15h respectively) but reaching wild type growth levels after 24h.

BHI and LB are still complex media that contain a mix of carbon sources that could interfere in our study of *L. monocytogenes* glucose-related growth. In order to determine the role of PTS and GlcU1 in the glucose uptake and the effect of their lack in the *in vitro* growth *of L. monocytogenes*, we decided to characterize the strains in the chemically-defined media IMM (Improved Minimal Media; Phan-Than and Gormon 1997) (Figure 10). Using this media turned out to be very variable from one experiment to another because every batch of IMM was giving different OD values. However, similar behaviour patterns of the strains were appreciated in each experiment.

The IMM assay corroborates previous results found in the routine medias, showing a much more marked difference in the growth of the glucose transporters mutants comparing with the rest of the strains when growing them in the presence of glucose as the only available carbon source (Figure 10A). Under these conditions, we saw that the wild type strain and the single mutant for the non-PTS glucose transporter *glcU1*::pAUL-A could reach maximum OD values of 1.8-2.0 after 22-26h. The growth of *ΔptsH* and *ΔptsH,glcU1*::pAUL-A strains was extremely delayed (14h and 24h respectively), to the point of not seeing *ΔptsH,glcU1*::pAUL-A reaching wild type levels even after 48h of incubation. However, by growing the *Listeria*

strains in IMM with uridine as an alternative carbon source (Figure 10B), we observed that all the strains (including mutants *ΔptsH* and *ΔptsH,glcU1*::pAUL-A) grew similarly reaching OD levels ≈ 0.6-08.

Our results probe that Δ*ptsH* and *ΔptsH,glcU1*::pAUL-A are able to metabolise other carbon sources as efficiently as the wild type does, discarding faults in the bacteria carbon metabolism and showing that the only explanation for the delay/decrease in the growth of these strains is their inefficient glucose transport.

No growth was observed for any of the *Listeria* strains when no source of carbon was added to the IMM (Figure 10C). That corroborates previous experiments in chemical defined media that showed the impossibility of *L. monocytogenes* to grow *in vitro* without a solid carbon source (Premaratne et al., 1991; Slaghuis *et al.,* 2007; Stoll 2008; Schneebeli and Egli, 2013).

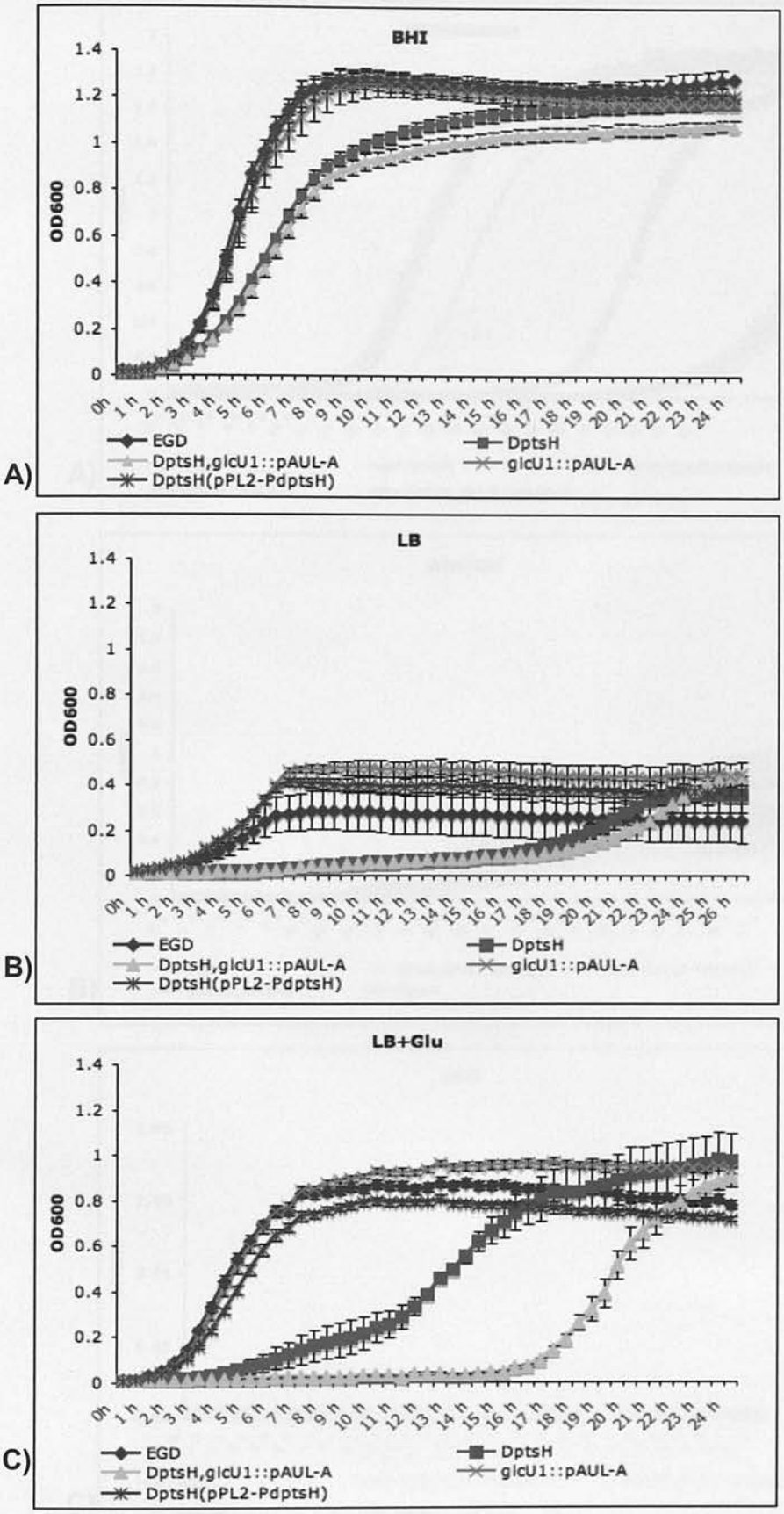

Figure 9. Representative *L. monocytogenes in vitro* growth curves in different medias: Strains EGD (wt), *ΔptsH, ΔptsH*(pPL2-P*δptsH)* ,*glcU1*::pAUL-A and *ΔptsH,glcU1*::pAUL-A were grown in BHI (A), LB (B) and LB supplemented with glucose (C), At least three independent experiments were performed for each strain (mean OD± SEM).

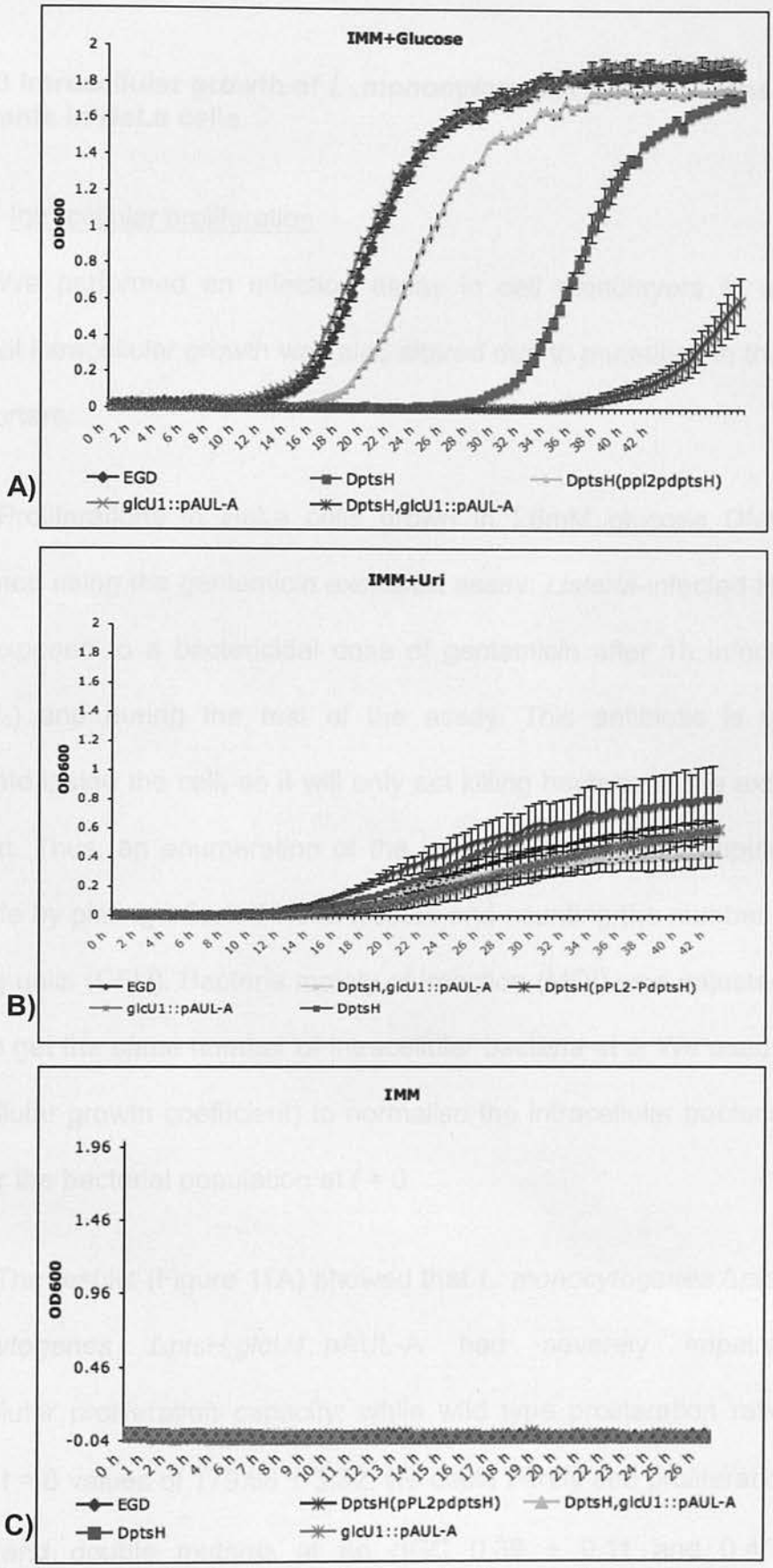

Figure 10. Representative *L. monocytogenes in vitro* growth curves in supplemented IMM: Strains EGD, *ΔptsH, ΔptsH*(pPL2-P*δptsH*) ,*glcU1*::pAUL-A and *ΔptsH,glcU1*::pAUL-A were grown in IMM supplemented with glucose (A), IMM supplemented with uridine (B) and IMM (C) for 48h.

2.1.3 Intracellular growth of *L .monocytogenes glucose* transporters mutants in HeLa cells

2.1.3.1 Intracellular proliferation

We performed an infection assay in cell monolayers to see if the bacterial intracellular growth was also altered due to mutations in the glucose transporters.

Proliferations in HeLa cells grown in 25mM glucose DMEM were performed using the gentamicin exclusion assay: *Listeria*-infected HeLa cells were exposed to a bactericidal dose of gentamicin after 1h infection (time zero, t_0) and during the rest of the assay. This antibiotic is unable to penetrate inside the cell, so it will only act killing bacteria in the extracellular medium. Thus, an enumeration of the intracellular bacterial population can be made by plating infected-HeLa lysates and counting the number of colony forming units (CFU). Bacteria moiety of infection (MOI) was adjusted in each case to get the same number of intracellular bacteria at t_0. We used IGC (the intracellular growth coefficient) to normalise the intracellular bacterial growth data for the bacterial population at $t = 0$.

The results (Figure 11A) showed that *L. monocytogenes* Δ*ptsH* and *L. monocytogenes* Δ*ptsH,glcU1*::pAUL-A had severely impaired their intracellular proliferation capacity: while wild type proliferation ratios reach IGC at $t = 8$ values of 179.68 ± 3.32, we could barely see proliferation for the single and double mutants at 8h (IGC 0.39 ± 0.11 and 0.45 ± 0.02 respectively). Generally *Listeria* intracellular proliferations are performed only

during 8h. However we wanted to check if Δ*ptsH* and Δ*ptsH,glcU1*::pAUL-A had the same growth behaviour as in *in vitro* conditions, showing a delay at 14h and 24h respectively but eventually reaching wild type intracellular levels.

At 48h (figure 11B), the difference between the wild type strain (IGC of 3448.1 ± 953.1), Δ*ptsH* (IGC of 75.2 ± 58) and Δ*ptsH,glcU1*::pAUL-A (IGC of 1.9 ± 1.8) was even bigger. Since our results showed strains lacking *ptsH* are affected in the intracellular growth, we decided to complement Δ*ptsH* to discard an impaired intracellular growth due to faults in another regions of the *Listeria* genome.

A Student's t-test was performed to analyse the proliferation data (figure 12) and only significant differences were found between the wild type and Δ*ptsH* and Δ*ptsH,glcU1*::pAUL-A ($p<0.05$ at 8h and $p<0.01$ at 24h). There were no significant differences when comparing any other tested strain with the wild type and also no differences were found in the proliferation capacity of Δ*ptsH* and Δ*ptsH,glcU1*::pAUL-A at any time ($t = 0.20$; $p=0.84$ at time 8h and $t = 1.6$; $p=0.15$ at 48h).

Taking into account the significant decrease in Δ*ptsH* and Δ*ptsH*,pAUL-A::*GlcU1* proliferation levels and previous experiments showing no fault in the bacteria virulence factors and neither in their carbon metabolism, we assumed that the decrease in the proliferation levels was caused by an inefficient glucose up-take.

However, It has been previously published that pathogenic *Listeria* requires being in the host cell cytosol to replicate and strains lacking functional PrfA and other virulence factors such as LLO have been shown to have an inefficient replication (and subsequently less proliferation levels) (Gedde *et al.,* 2000, De las Heras *et al.,* 2011). We then decided to microscopically test Δ*ptsH* and Δ*ptsH*,pAUL-A::*GlcU1* to check their ability to escape from the phagosome reaching the replicative niche .

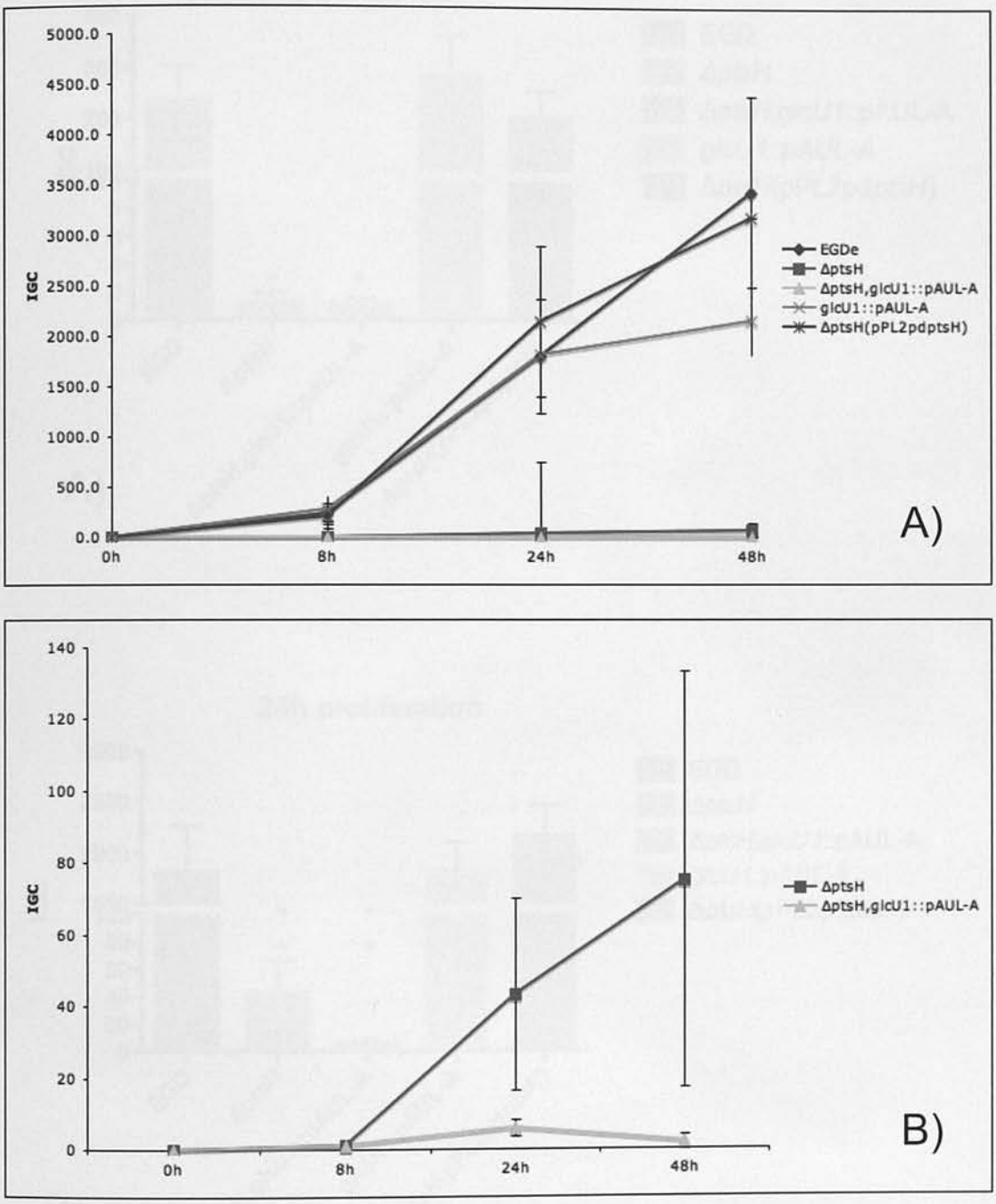

Figure 11. Intracellular proliferation of *L. monocytogenes* EGD, in HeLa cells.
IGC values for wild type EGD, *glcU1*::pAUL-A, Δ*ptsH*, Δ*ptsH*(pPL2-*PδptsH*) *and* Δ*ptsH*,*glcU1*::pAUL-A in a 48h proliferation assay (A). Focused IGC values for Δ*ptsH* and Δ*ptsH*,*glcU1*::pAUL-A are shown in figure B. Data are mean values of four independent experiments ± SE.

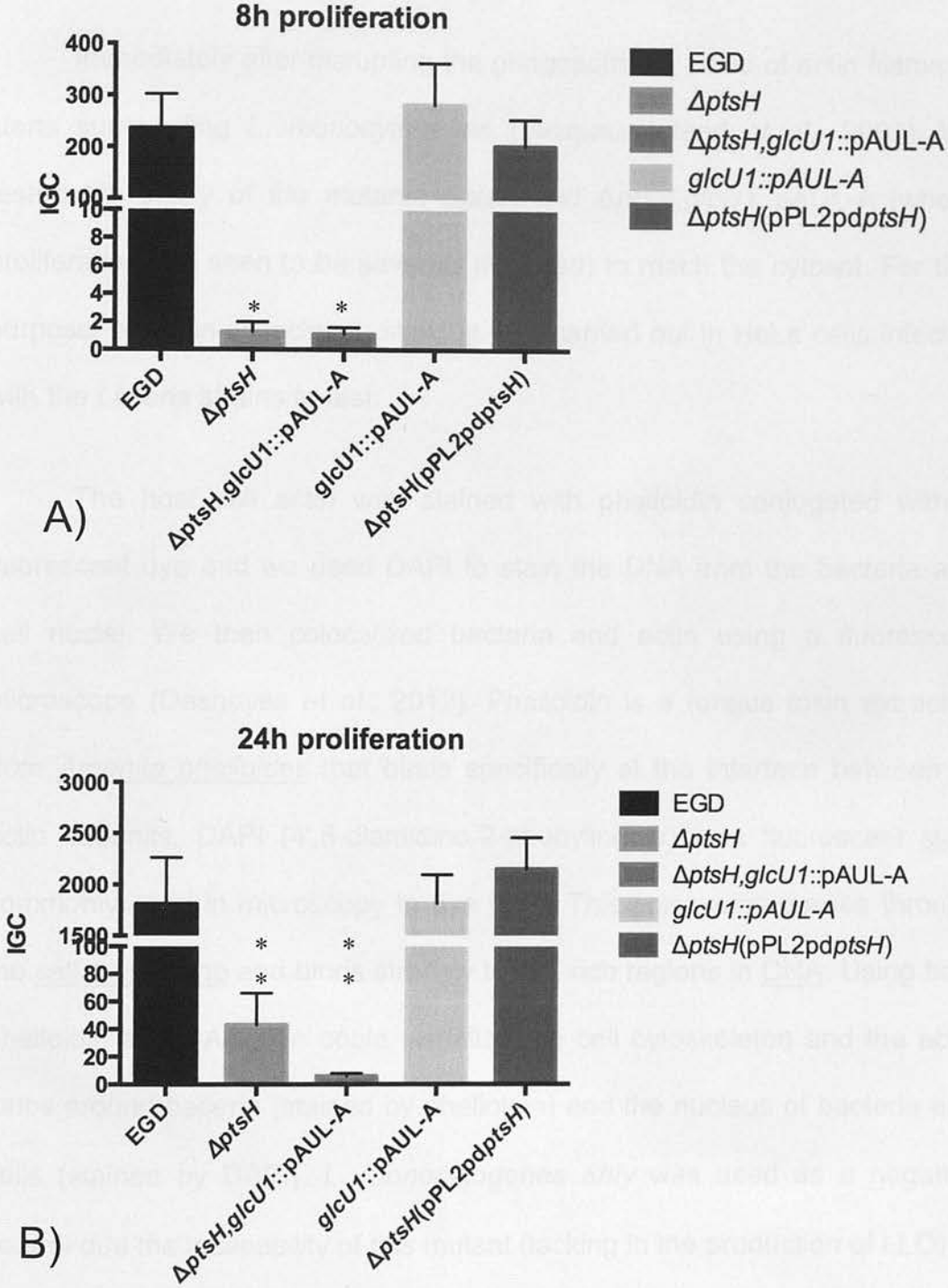

Figure 12. Statistical analysis for L. monocytogenes proliferation in HeLa cells. Multiple Student's t tests for the IGC values were performed at 8h (A) and 48h (B). Data are mean values of at least three independent experiments ± SE. (*) means t-values with a $p<0.05$. (**) means t-values with a $p<0.01$.

2.1.3.2 Visualization of cytosolic bacteria

Immediately after disrupting the phagosome, a cloud of actin filaments starts surrounding *L. monocytogenes* (Vazquez-Boland *et al.,* 2001). We tested the ability of the mutants Δ*ptsH* and Δ*ptsH,glcU1*::pAUL-A (whose proliferation has seen to be severely impaired) to reach the cytosol. For that purpose, an actin-association staining was carried out in HeLa cells infected with the *Listeria* strains to test.

The host cell actin was stained with phalloidin conjugated with a fluorescent dye and we used DAPI to stain the DNA from the bacteria and cell nuclei. We then colocalized bacteria and actin using a fluorescent microscope (Deshayes *et al.,* 2012). Phalloidin is a fungus toxin extracted from *Amanita phalloides* that binds specifically at the interface between F-actin subunits. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain commonly used in microscopy to dye DNA. This compound passes through the cell membrane and binds strongly to A-T rich regions in DNA. Using both phalloidin and DAPI, we could visualize the cell cytoskeleton and the actin halos around baceria (stained by phalloidin) and the nucleus of bacteria and cells (stained by DAPI). *L. monocytogenes* Δ*hly* was used as a negative control due the incapability of this mutant (lacking in the production of LLO) to escape efficiently from the vacuole (Gedde *et al.,* 2000).

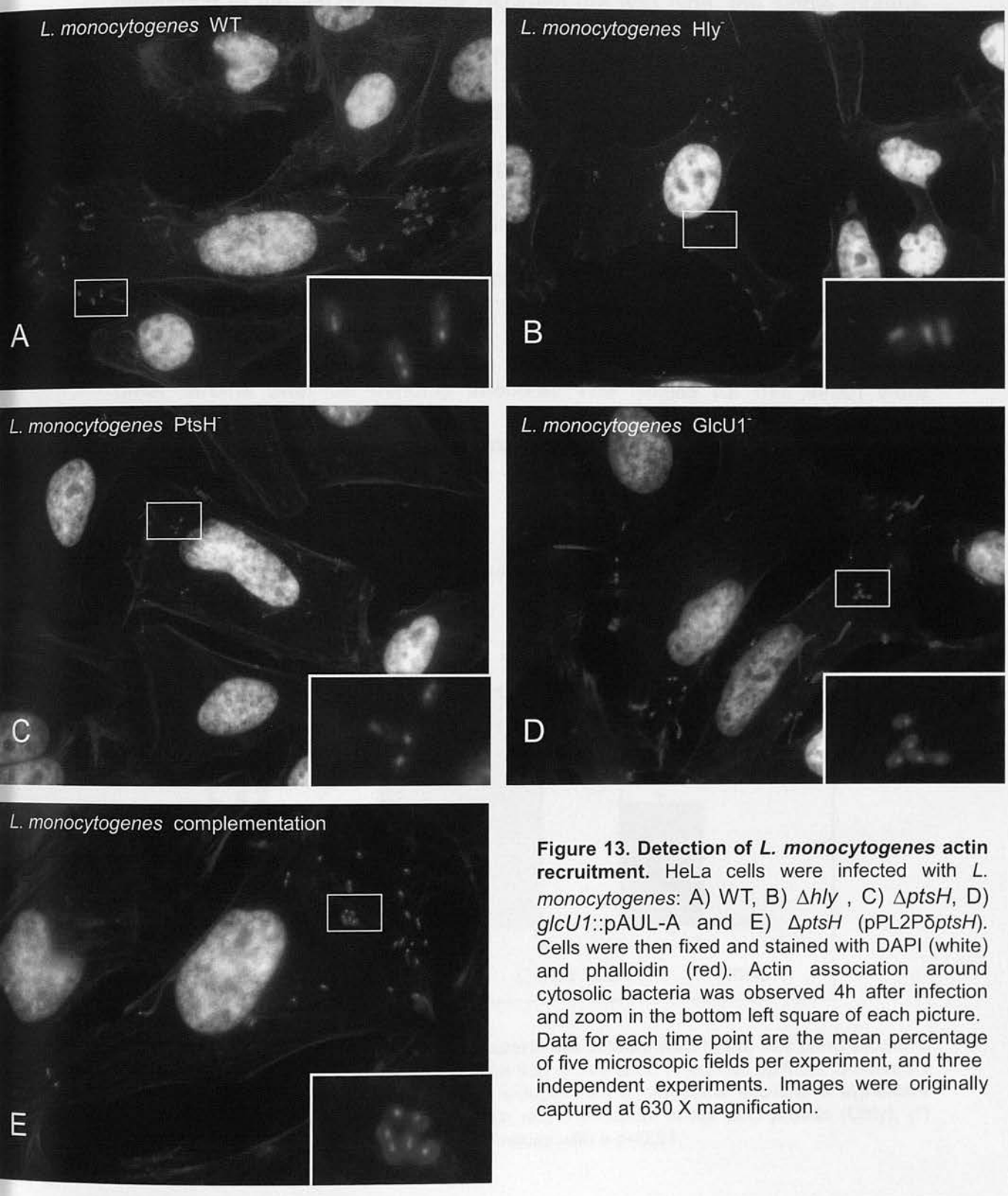

Figure 13. Detection of *L. monocytogenes* actin recruitment. HeLa cells were infected with *L. monocytogenes*: A) WT, B) Δ*hly* , C) Δ*ptsH*, D) *glcU1*::pAUL-A and E) Δ*ptsH* (pPL2Pδ*ptsH*). Cells were then fixed and stained with DAPI (white) and phalloidin (red). Actin association around cytosolic bacteria was observed 4h after infection and zoom in the bottom left square of each picture. Data for each time point are the mean percentage of five microscopic fields per experiment, and three independent experiments. Images were originally captured at 630 X magnification.

The images (figure 13 and 14) showed that there is not a significant difference in the vacuole escape between the wild type, the single mutants Δ*ptsH and glcU1*::pAUL-A and the complemented strain Δ*ptsH*(pPL2-Pδ*ptsH*), suggesting that Δ*ptsH* had the same escape and actin recruitment capacity as the wild type phenotype. Although we were not able to find intracellular Δ*ptsH,glcU1*::pAUL-A bacteria at the time of the measurement, the proliferation assay probed the existence of intracellular Δ*ptsH,glcU1*::pAUL-A, showing the same number of initial bacteria as the other strains after 30 minutes infection. The results for this strain were inconclusive, suggesting that the experimental conditions should be further optimised.

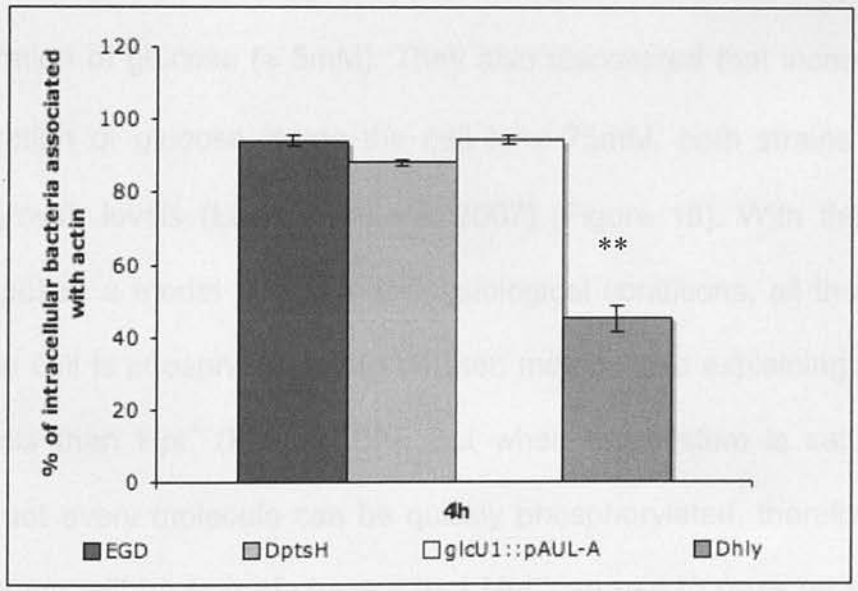

Figure 14. Percentage of intracellular bacteria associated with actin. The panel presents the quantification of the intracellular bacteria that recruit actin (mean%± SEM%). Student's t tests were performed on the results of 3 independent experiments showing a significant difference only between the wild type strain and the mutant in the LLO protein (Dhly). (*) means t-values with a $p<0.05$. (**) means t-values with a $p<0.01$.

Glucose dependence of *L. monocytogenes* intracellular growth

Our previous results are a direct evidence of the importance of glucose in the intracellular metabolism of *L. monocytogenes*. In order to provide new evidence about the essential role of free intracellular glucose in *L. monocytogenes* growth, we decided to use a different strategy modifying the conditions inside the HeLa cells (host model) instead of using *L. monocytogenes* glucose uptake defective strains.

Previous experiments in Vazquez-Boland group (Lecharme-Lora, 2007) determined that there was a growth difference between the wild type strain (Hpt^+) and the mutant in the HPs transporter (Hpt^-) at physiological concentration of glucose (≈ 5mM). They also discovered that increasing the concentration of glucose inside the cell to ≈ 25mM, both strains reached similar growth levels (Lecharme-Lora, 2007) (Figure 15). With these data, they proposed a model in which at physiological conditions, all the glucose inside the cell is phosphorylated to be then metabolised explaining why Hpt^- grows less than Hpt^+ (Figure 15A). But when the system is saturated of glucose not every molecule can be quickly phosphorylated, therefore inside the cell there will be free of glucose that Hpt^- can use to grow on the same way the wild type Hpt^+ does (Figure 15B).

In order to obtain a pool of free glucose inside the cell at physiological conditions, they decided to deplete HeLa cells hexokinases (HK) by using an

non-specific inhibitor: 3-bromopyruvate. HKs phosphorylate glucose forming G6P as one of the first steps of the glycoltic pathway. Inhibiting the action of these enzymes, no glucose will be broken down and it will accumulate inside the cell. Then, they performed a parallel experiment using both strains Hpt^+ and Hpt^- in physiological conditions with and without the inhibitor.

Results for that experiment show that while the Hpt^- growth had increased in the presence of the inhibitor, Hpt^+ growth had decreased (figure 15C). That suggests that in the absence of G6P, the wild type doesn't have the advantage of an additional source of carbon for a rapid intracellular growth, therefore its intracellular proliferation values are lower than in normal cellular conditions. On the other hand, Hpt^- that in normal conditions doesn't have enough intracellular glucose to reach wild type proliferation levels, finds a high glucose-concentrated cytoplasm that can use to meet its nutritious requirements nearly reaching wild type intracellular growth levels (figure 15C). However, there is still a difference between the Hpt^+ and Hpt^- intracellular growth in the presence of the inhibitor, what drove them to think that the inhibition of the HKs could have been incomplete, thus less free glucose was available in the cytosol for Hpt^- to use it.

In this project we sought to see if there is still a difference between Hpt^+ and Hpt^- intracellular replication in cells that have been HK-silenced by using a specific inhibitor. Small interference RNA (siRNA) was selected to deplete HK expression due to the specificity of the method.

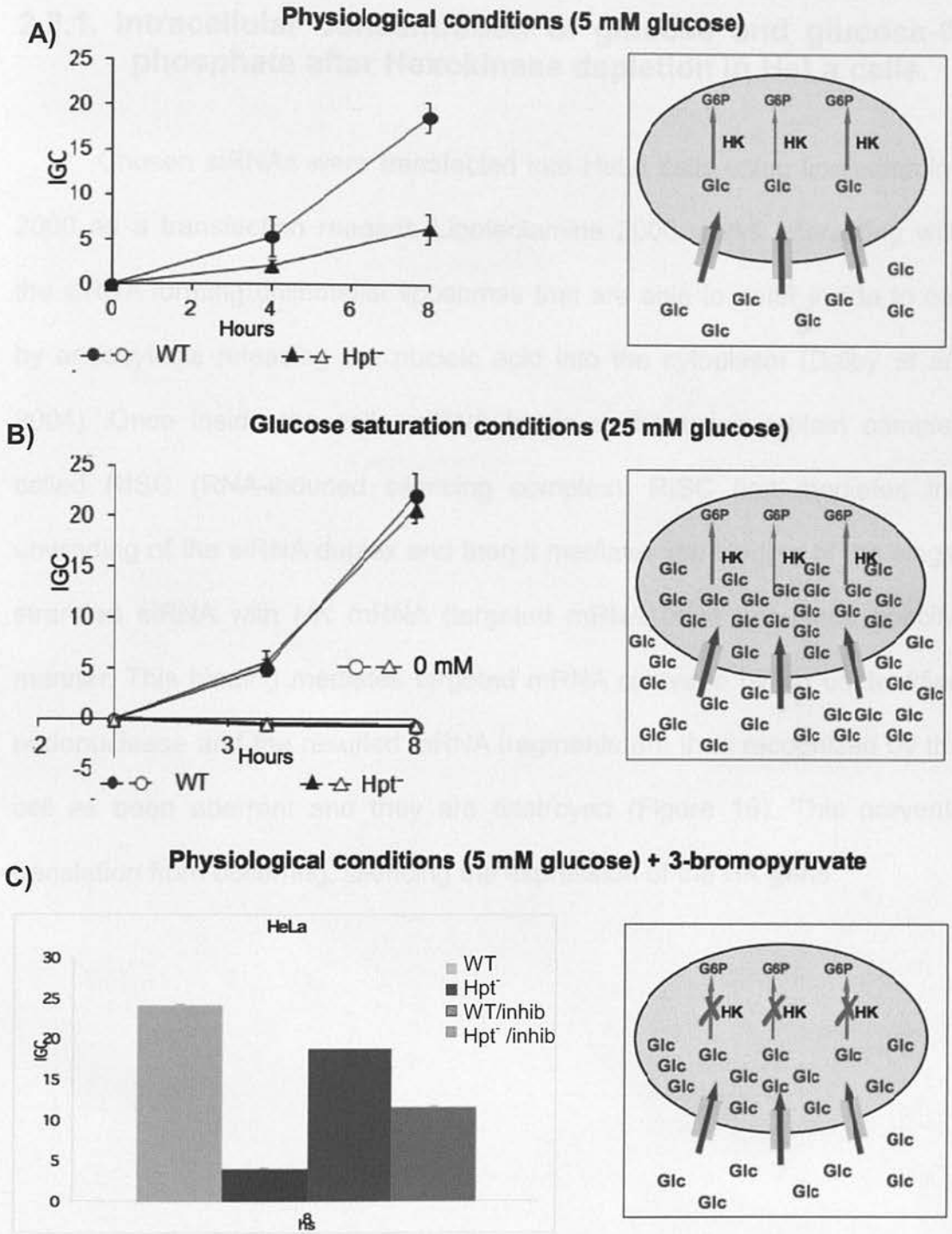

Figure 15: Intracellular growth of *L. monoctogenes* depends on glucose and G6P availability. A) At physiological conditions HeLa cells uptake and immediately transform glucose into G6P (right panel, schematic representation). In this situation *L.monocytogenes* Hpt^- grows less than wild type Hpt^+ (left chart). B) When the system is saturated of glucose not every glucose molecule can be transformed and an intracellular pool of free glucose appears (right panel, schematic representation). Then, Hpt^- and Hpt^+ use that glucose as a carbon source growing at the same level (left chart). C) Using the HK inespecific inhibitor: 3-bromopyruvate a intracellular pool of glucose was generated at physiological conditions (right panel, schematic representation). Hpt^- growth increases in the presence of the inhibitor, while Hpt^+ growth decreases (left chart). (Lecharme-Lora, 2007)

2.2.1. Intracellular concentration of glucose and glucose-6-phosphate after Hexokinase depletion in HeLa cells.

Chosen siRNAs were transfected into HeLa cells using lipofectamine 2000 as a transfection reagent. Lipofectamine 2000 works interacting with the siRNA forming unilamellar liposomes that are able to enter inside to cell by endocytosis releasing the nucleic acid into the cytoplasm (Dalby *et al.*, 2004). Once inside the cells, siRNA forms a ribonucleic-protein complex called RISC (RNA-induced silencing complex). RISC first mediates the unwinding of the siRNA duplex and then it mediates the binding of the single stranded siRNA with HK mRNA (targeted mRNA) in a sequence specific manner. This binding mediates targeted mRNA cleavage by an unidentified endonuclease and the resulted mRNA fragments are then recognized by the cell as been aberrant and they are destroyed (Figure 16). This prevents translation from occurring, silencing the expression of the HK gene.

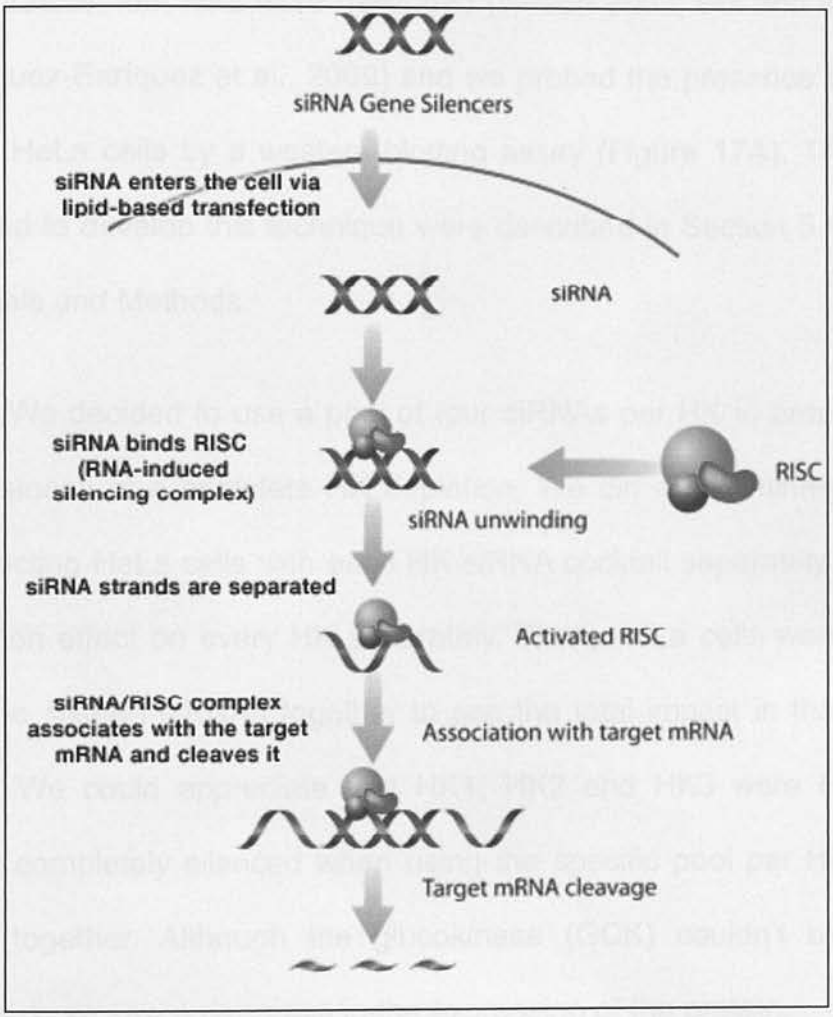

Figure 16. Schematic representation of the siRNA action (Santa Cruz biotechnology, inc)

2.2.1.1 Optimisation of the HK depletion using siRNA

A bibliographical search was performed in order to establish the number of HKs that have been described and characterized up to the date. in the literature, four HKs were described (Wilson 2003; Sui and Wilson, 2004; Rodríguez-Enríquez et al., 2009) and we probed the presence of all of them in our HeLa cells by a western blotting assay (Figure 17A). The antibodies required to develop this technique were described in Section 5.4.5 (Table 5), Materials and Methods.

We decided to use a pool of four siRNAs per HK in order to increase the chances of a complete HK depletion. We did a preliminary experiment transfecting HeLa cells with each HK-siRNA cocktail separately to check the depletion effect on every HK separately. Then, HeLa cells were transfected with the sixteen siRNAs together to see the total impact in the cells (figure 17.B). We could appreciate that HK1, HK2 and HK3 were completely or nearly completely silenced when using the specific pool per HK or the four pools together. Although the glucokinase (GCK) couldn't be completely depleted, we saw a decrease in the expression of the protein.

Being sure about the effect of the siRNA on the cells, we decided to investigate the proliferation of wild type *L. monocytogenes* (Hpt^+) and the mutant lacking functional Hpt (Hpt^-) in siRNA-depleted HeLa cells. The experimental set-up consisted of obtaining a balance in which at ≈5mM glucose media (physiological conditions), the cells appeared healthy whilst the siRNA was effective. To achieve this, we tested different conditions

(Figure 18). We finally performed a 4h transfection in standard DMEM media (25 mM glucose) followed by a media replacement, using DMEM 5mM glucose for the rest of the experiment. The proliferation assay lasted for 6h.

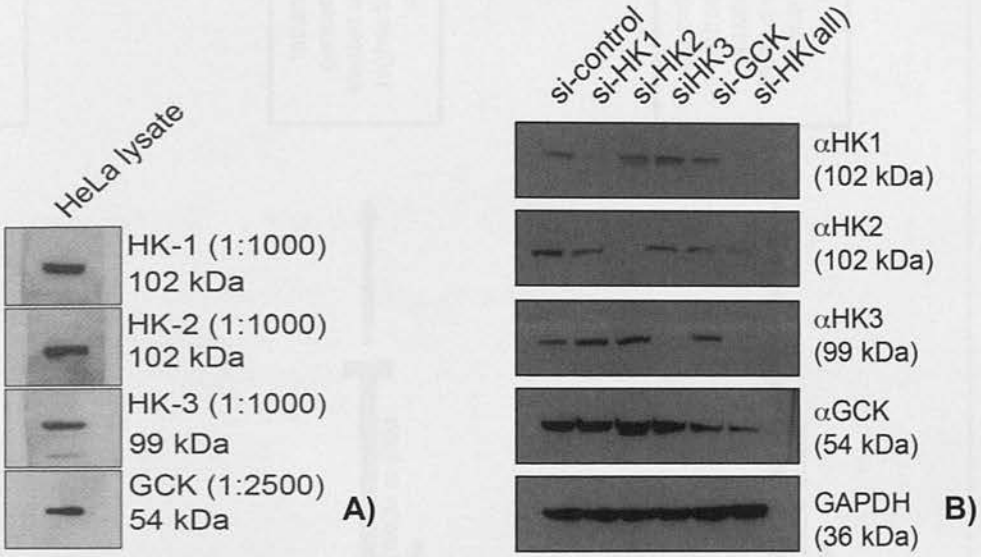

Figure 17. Detection of the glucokinase (GCK) and hexokinase (HK) proteins by western blot: A) Detection of the HK1, HK2, HK3 and GCK in HeLa cell extracts. The concentration of antibody used and the protein weight are specified in each case. A) Detection of the siRNA action: Each HK was individually targeted using four different siRNA (gel lanes two to four); gel lane five is a combination of the 16 siRNAs use in total to target the four HKs present in HeLa cells simultaneously.

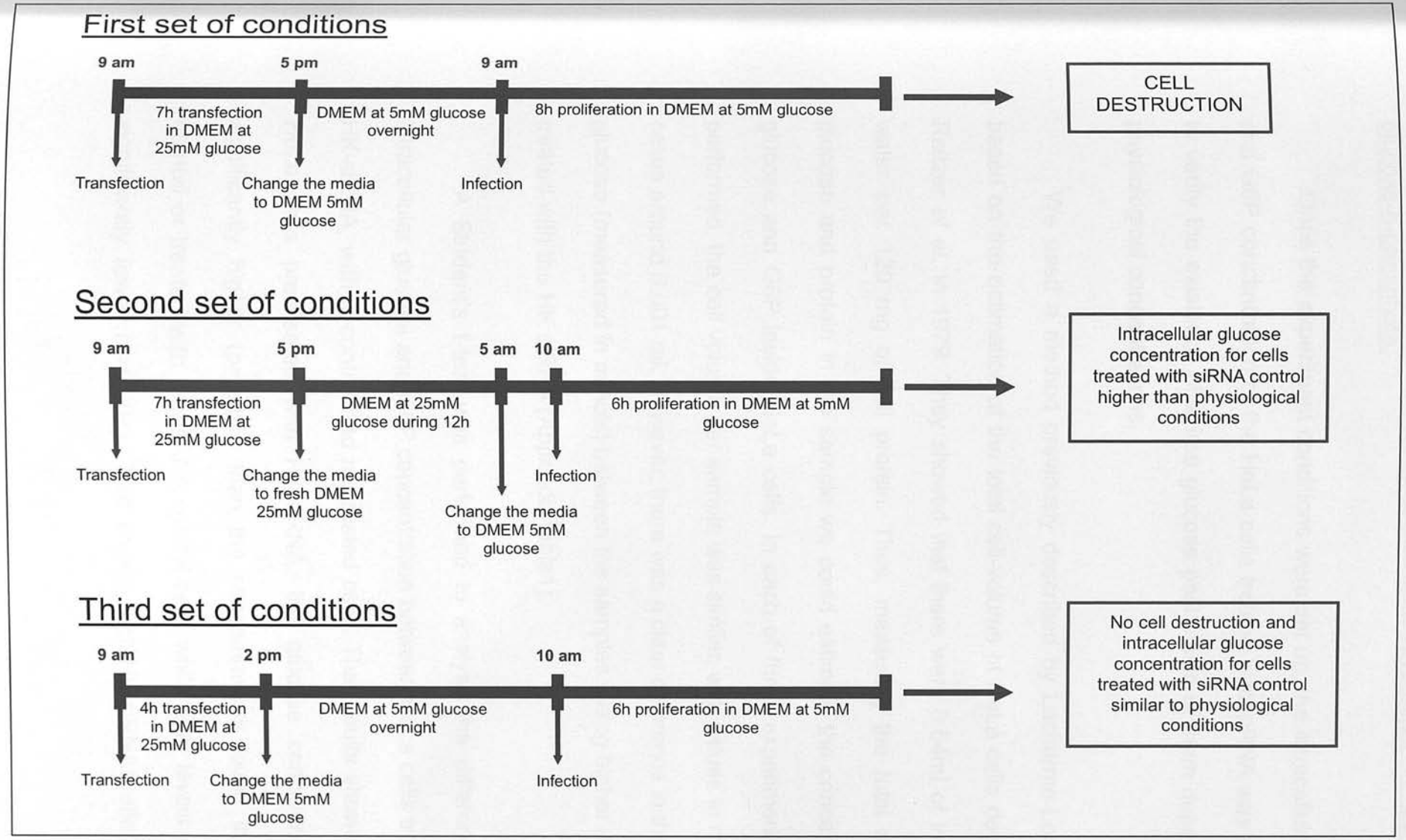

Figure 18. Schematic representation of the different strategies that we have followed to set up the *L .monocytogenes* proliferation assay in siRNA transfected HeLa cells. A time line is represented in red and the results for each trial are framed into a red square.

2.2.1.2 Determination of the intracellular concentration of glucose and glucose-6-phosphate.

Once the experiment conditions were set up, the intracellular glucose and G6P concentration of the HeLa cells treated with siRNA was measured to verify the existence of a free glucose pool inside of them departing from physiological concentrations.

We used a method previously described by Lacharme-Lora (2005), based on the estimation of the total cell-volume of HeLa cells described by Reitzer *et al.* in 1979. They showed that there were 0.64ml of intracellular water per 120 mg of cell protein. Thus, measuring the total amount of glucose and protein in the sample we could estimate the concentration of glucose and G6P inside HeLa cells. In each of three experiments that we performed, the cell volume per sample was similar, with values in most of the cases around 0.001 ml. However, there was a clear difference in the levels of glucose (measured in mmols) between the samples, being higher in the cells treated with the HK siRNA (Appendix table1).

A Student's t-test was performed to analyse the difference in the intracellular glucose and G6P concentration between HeLa cells treated with HK-siRNA, with si-control and no treated cells. The results showed that for HeLa cells pre-treated with HK-siRNA, the glucose concentration was significantly higher ($p=0.018$) than the concentrations found in both no treated or treated with the siRNA control cells while G6P levels were very significantly lower ($p=0.007$). That shows that HK-siRNAs silenced HKs

expression avoiding the phosphorylation of the glucose into G6P and creating in turn an accumulation of glucose inside the cells.

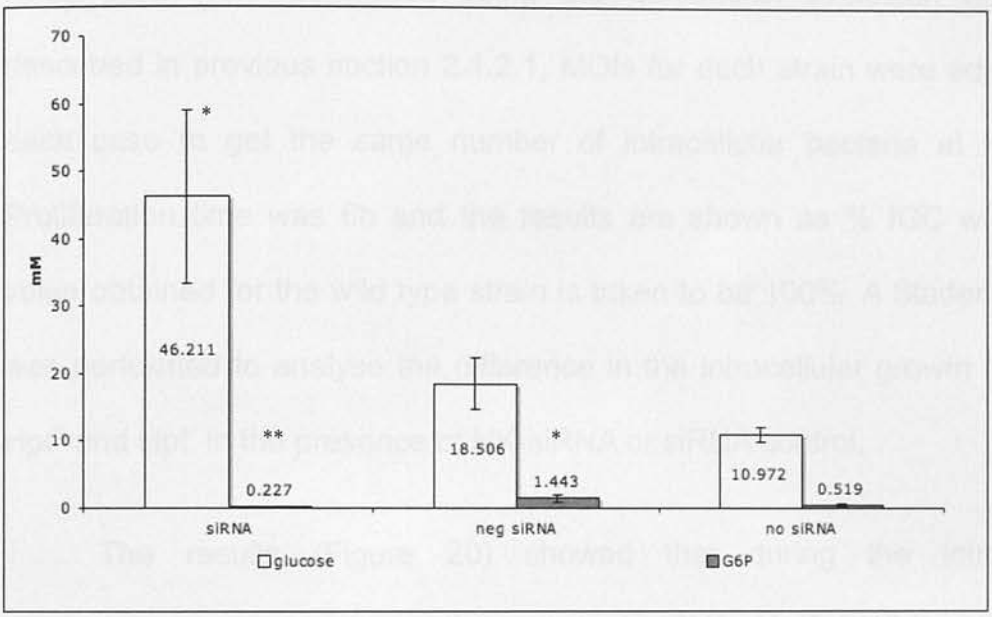

Figure 19. Levels of glucose and G6P inside HeLa cells pre-treated with HK siRNA (siRNA). The figure shows concentration (expressed in mM) of glucose and G6P expressed (mean ± SEM) in HeLa cells treated with a commercial siRNA control (neg siRNA) and non-treated (no siRNA), were used as a controls for the experiment. Student's t tests were performed on the results of 3 independent experiments showing significant (*, $p<0.05$) and very significant (**, $p<0.001$) differences in the glucose and G6P concentration of HeLa treated with siRNA comparing with untreated cells and cells treated with the siRNA control.

2.2.2. Effect of the intracellular glucose concentration on Hpt-dependent listerial growth in HeLa cells.

Finally, HeLa cells pre-treated with siRNA infected with wild type *L. monocytogenes* (Hpt^+) and the mutated strain (Hpt^-). *Listeria* proliferationsin HeLa cells were performed using the gentamicin exclusion assay as described in previous section 2.1.2.1, MOIs for each strain were adjusted in each case to get the same number of intracellular bacteria at time 0h. Proliferation time was 6h and the results are shown as % IGC where the value obtained for the wild type strain is taken to be 100%. A Student's t-test was performed to analyse the difference in the intracellular growth between Hpt^+ and Hpt^- in the presence of HK-siRNA or siRNA control.

The results (Figure 20) showed that during the intracellular proliferation in the presence of the control siRNA, Hpt^+ (%IGC at t=8 99.5 ± 16.7) grew significantly more ($p=0.0047$) than Hpt^- (%IGC 37.7 ± 9.4). However, when the cells were treated with HK-siRNA, Hpt^- showed wild type growth levels (%IGC at t=8 106± 17.1).

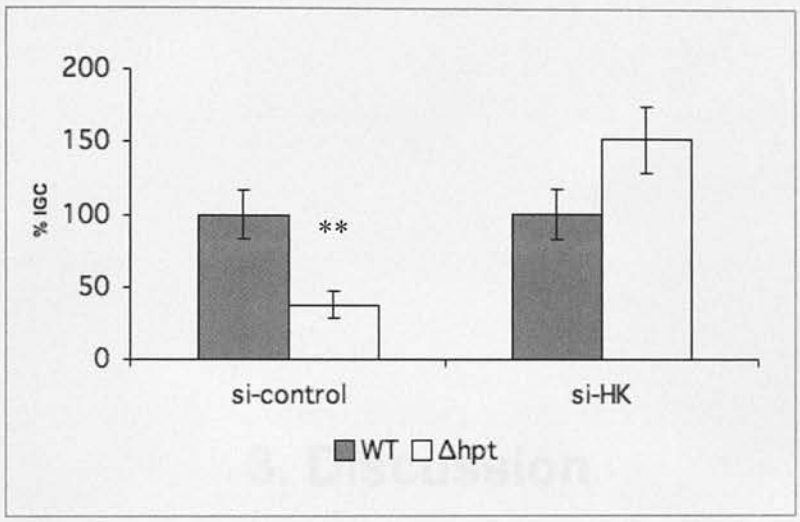

Figure 20. 6h Intracellular proliferation of *L.monocytogenes* in HeLa cells pre-treated with HK siRNA (si-HK). Hpt^{+} (WT) appears in grey and *Hpt*⁻ appears in white. HeLa pre-treted with a commercial siRNA control (si-control) were used as a controls for the experiment. Mean of three independent experiments ± SEM. results are shown as % IGC where the value obtain for the wild type strain is taken to be 100%. A Student's t-test was performed to analyse the data. (*) means t-values with a $p<0.05$. (**) means t-values with a $p<0.01$.

3. Discussion

3.1 Discussion

Intracellular parasites adapt their metabolism to the host cell life cycle in order to survive and proliferate. The understanding of metabolic changes has become an active area of research, as interfering in the pathogen nutrient-uptake process could mean a new and more effective way of treating the disease (Muñoz-Elias and McKinney, 2006; Eisenreich et al., 2010).

Every pathogen is different and so is the way it interacts with its host cells. The intracellular pathogen *M. tuberculosis* completely changes its nutritional requirements on entry into the host. Intracellular *M. tuberculosis* utilises fatty acids as a carbon source, in contrast its *in vitro* growth is based on a carbohydrate metabolism (Weddel, 2010; Russell, 2011). The Food-borne pathogen *S. typhimurium* secretes a set of effector proteins into the host modifying the cell functions for its own benefit (Agbor & McCormick, 2013). Although glucose has been described to be its preferred extra- and intracellular carbon source, *S. typhimurium* is capable of switching to the glycerol metabolism pathway when required (Goebel and Gotz, 2010; Kim et al., 2013).

It is well known that *Listeria* uses glucose or an early metabolite of the glycolitic pathway as its main carbon source under extracellular conditions (Tsai and Hodgson, 2003; Schneebeli and Egli, 2013). However, intracellular *Listeria* parasitises the host cell metabolism using hexose-sugars already phosphorylated by the host. Chico-Calero (2002) and collaborators described these hexose –phosphates as extra fuel for efficient intracellular proliferation,

but not essential for *Listeria* intracellular replication. Other carbon sources such as glycerol (Joseph *et al.,* 2008) or glucose (Lacharme-Lora, 2007) have been proposed as the main intracellular source of carbon for the bacteria.

Previous results from Vazquez-Boland laboratory suggested an important role of glucose in *Listeria* intracellular proliferation (Lacharme-Lora, 2007). In order to clarify the real effect of cytosolic free glucose in listerial intracellular replication, we carried out experiments using *L. monocytogenes* mutants defective in glucose high/low-affinity transporters PTS system and GlcU1 permease.

Glucose up-take through the PTS system is not specific to *Listeria* but occurs often among bacteria (Barabote and Saier, 2005). Other food-borne pathogens such as the enteroinvasive *E. coli* (EIEC), *Shigella flexn (S. flexineri)* (that act similarly to *Listeria* escaping from the phagosome to actively replicate in the cytoplasm) also use PTS as main glucose up-take system under intra/extracellular conditions (Gotz and Goebel, 2010). An insertion mutant defective in the *ptsH* gen (enconding Hpr protein, a central component of all PTS pathways) was described by Mertins *et al.* (2007). Their in vitro experiments measuring the effect of the PTS- mutation on the virulence genes PrfA-regulated, showed an induction of those genes when the glucose uptake via PTS was impaired. Stoll and collaborators (2008) supported Mertins et al.'s findings suggesting that PTS sugars had an inhibiting effect on the PrfA under in vitro conditions. However, in our

experiments proliferating *L. monocytogenes* in HK-depleted HeLa cells, there was no significant difference proliferating listeria in cells with a high intracellular glucose concentration (due to a siRNA HK-depletion), comparing with the proliferation levels in cells at physiological conditions. We have also proliferated ΔptsH inside HeLa cells and our results showed a high significant decrease in the growth levels of the mutant compared with the parental strain, suggesting that listeria requires glucose for its intracellular replication.

We described for the first time a double mutant lacking both PTS and GlcU1 (non-PTS glucose pearmease). The proliferation of this strain in HeLa cells was (as ΔptsH intracelluar growth) completely impaired. This corroborates our previous result obtained with ΔptsH and is direct evidence of the importance of glucose for intracellular Listeria. The proliferation of the single mutant for the GlcU1 pearmeases was the same as the wild type; only the strains lacking ptsh were affected in the intracellular growth. Therefore, based on this evidence we propose that the PTS system is the main glucose transporter for intracellular listeria. Our *in vitro* characterization of the single mutants for PTS and GlcU1 permeases and the double mutant PTS^- $GlcU1^-$ determined that the PTS system is the main glucose transporter also in vitro corroborating previous results of (Stoll and Goebel, 2010). When the glucose uptake through the PTS system is active, GlcU1 function is hidden. However when the bacteria are defective in both transporters, PTS cannot balance the lack of GlcU1 and the activity of the low-affinity glucose transporter comes to light.

To provide further evidence for this conclusion from the host cell side, we proliferated Hpt^- (mutant laking hpt) in HK-depleted host cells. The mutant (unable to use HP) showed wild type growth in HK-depleted HeLa cells. This is indirect evidence for the previous conclusion that glucose (before being phosphorylated) is a major carbon source for intracellular *L. monocytogenes.*

In conclusion, the results from the proliferation in knock down cells in addition with the glucose-uptake mutant experiments indicate that glucose (before its conversion into G6P) is an essential carbon source for intracellular *L. monocytogenes* replication. Moreover, our results show that there is no difference in *L. monocytogenes* carbon metabolism between intra- and extracellular conditions, behaving similarly to other intracellular pathogens with an active cytosolic replication (Gotz et al, 2010). This is also concordant with analyses of the *Listeria* genome that show no substantial metabolic differences between environmental strains and pathogenic strains that have evolved an intracellular life-cycle (Iliffe-Lee and McClarty 2000).

4. Conclusions and future work

4.1 Conclusions

The experimental work described in this project has led to the following conclusions:

(i) We *in vitro* characterized Δ*ptsH* confirming previous observations from other groups that determine PTS system as the main *in vitro* glucose transporter for *L. monocytogenes.* We have also studied Δ*ptsH* intracellular replication demonstrating the role of the PTS system as the principal intracellular glucose up-taker.

(ii) We built, characterized and studied the intracellular growth of two *L. monocytogenes* strains defective in the most important glucose transport systems for the bacteria: pAUL-A::*glcU1* defective in the low-affinity non-PTS up-taker GlcU1 and Δ*ptsH,*pAUL-A::*glcU1* defective in both PTS and non-PTS transporters. Our results support an important role of GlcU1 as a non-PTS glucose transporter *in vitro,* whose action is clearly reflected in the absence of the PTS system. We could appreciate some intracellular glucose up-take through GlcU1, however the contribution of this transporter *in vivo* is not as important as its function *in vitro.*

(iii) Our work with Δ*ptsH*, pAUL-A::*glcU1* and Δ*ptsH,*pAUL-A::*glcU1* provides strong experimental support to the notion that *L. monocytogenes* uses mainly glucose as a carbon source in *in vitro* conditions and when parasiting a cell.

(iv) We identified four HK in HeLa cells, HK1, HK2, HK3 and GCK

and we have demonstrated the efficiency of the selected siRNA depleting the action of theses intracellular proteins.

(v) We confirm previous observations from our laboratory that suggested that *L. monocytogenes* deficient in the HP transport was able to use free intracellular glucose showing wild type growth. These results indirectly support the idea of glucose as a sufficient and essential carbon source for intracellular *L. monocytogenes*.

4.2 Future work

Future directions of the research based on the findings and work reported in this project are:

(i) To investigate the effect of the complete deprivation of intercellular glucose and G6P for the bacteria in order to definitely determine which carbon sources are essential for the intracellular parasitic *L. monocytogenes*. For that purpose the construction, characterization and a intracellular behaviour study of double and triple mutants in the Hpt transporter and the PTS system, as well as combined with the non-PTS sugar transporter GlcU1 will be the next step to follow.

(ii) Future work will also focus on the construction, characterization and intracellular growth study of single mutants for the other two non-PTS glucose transporters GlcU2 and GlcU3 and double mutants combining them with for the mutation in the PTS system, in order to

determine the function of GlcU2 and GlcU3 in *L. monocytogenes* intracellular glucose transport. It will be also a priority in our research, a further optimisation of the experimental conditions for the visualization of intracellular *L. monocytogenes* PTS^- $GlcU1^-$.

(iii) It will also be interesting to investigate the behaviour of the mutant strains PTS^- and PTS^- $GlcU1^-$, already characterized and studied, in an *in vivo* mouse model, to see if the bacteria follows the same pattern in a *in vivo* infection.

(iv) Seen the results achieved doing proliferations of *L. monocytogenes* wild type and Δ*hpt* in HK-depleted HeLa cells, future work will also focus on adapting the siRNA knockdown assay to a mouse model in order to investigate how Hpt^- behaves in an *in vivo* proliferation.

5. Materials and Methods

5.1 Microbiological techniques

5.1.1 Bacterial strains and culture conditions

L. monocytogenes serovar 1/2a strain EGD-e (Glasser *et al.,* 2001), serovar 4b strain P14 (Rippio et al., 1997) and *E. coli* DH5α (Hanahan, 1985) were used to carry out the experiments (Table 1). *Listeria* strains were routinely cultured in BHI (Difco Laboratories-Beckto). *L. monocytogenes* mutants deficient in the uptake of glucose were grown in LB (Sigma) supplemented with 20mM of uridine as a carbon source (Sigma). *E. coli* strains were routinely cultured in LB media. In order to prepare solid growth media, 1.6 % of bacteriological agar (Oxoid) was added to the broth media. *Listeria* was grown at 37°C (shaking at 200 rpm for liquid cultures). Media was supplemented with erythromycin (Em) or chloramphenicol (Cm)(Sigma) when it was required.

5.1.2 *Listeria* chemical defined media preparation

IMM was prepared according to Phan-Than & Gormon (1997) with modifications (Table 2). IMM medium was stored at 4°C for periods of no longer than two weeks and protected from light. Selected carbon source was added to the media at 20mM concentration as required.

Strains	Description	Reference	Collection no.
L. monocytogenes			
EGDe	*Listeria monocytogenes* serovar 1/2a	Glasser, 2001	PAM 915
ΔptsH	Derivative strain of EGD-e obtained by in-frame deletion of the *ptsH* gene	Scortti *et al.* (unpublished)	PAM 1019
pAUL-A::glcU1	Derivative strain of EGD-e obtained by insertion of pAUL-A plasmid in *glcU1* gene	This project	PAM 3808
ΔptsH,pAUL-A::glcU1	Derivative strain of EGD-e*ΔptsH* obtained by in-frame insertion of pAUL-A plasmid in *glcU1* gene	This project	PAM 3810
ΔptsH,pPL2-PδptsH	Derivative strain of EGD-eΔptsH obtained by complementing the strain with the integrative plasmid pPL2Pδ	This project	PAM 3811
P14	*Listeria monocytogenes* serovar 4b	Rippio *et al.*, 1997a	PAM 14
ΔprfA	Derivative strain of EGD-e obtained by deletion of the *PrfA* gene	Mengaud et al., 1991	PAM 374
Δhly	Derivative strain of P14 obtained by in-frame deletion of the *ptsH* gene	De las Heras. (unpublished)	PAM 3674
P14A	*Listeria monocytogenes* serovar 4b with PrfA constitutively up-regulated	Rippio *et al.*, 1997a	PAM 50
Δhpt	Derivative strain of P14A obtained by in-frame deletion of the *hpt* gene	Chico-Calero *et al.*, 2002a	PAM 377
E. coli			
DH5α	Cloning host strain	Hanahan, 1985	PAM 184
Plasmids			
pCR Blunt	Cloning plasmid for blunt PCR fragments. Plasmid carries Kanamycin and Zeocin resistance genes.	Invitrogen	-
pAUL-A	Cloning plasmid for gene replacement in *Listeria.* Plasmid carries Em resistance gene.	Chakraborty , 1992	PAM 3175
pAUL-A*glcU1*	pAUL-A derivative containing *glcU1* gene	This project	PAM 3806
pPL2Pδ	Site specific *Listeria* mutagenesis vector. Plasmid carries Cm resistance.	De las Heras (unpublished)	PAM 3574
PL2Pδ*ptsH*	pPL2Pδ derivative containing *ptsH* gene expressed from the highly constitutive promoter Pδ	This project	PAM 3812

Table 1. Bacterial strains and plasmids used in this study.

Compound		Stock Solution	Volume of stock per Litre of IMM
Phosphates solution	KH_2PO_4	6,56 g/L	100 ml
	Na_2HPO_4* 7 H_2O	30,96 g/L	
$MgSO_4$ * 7 H_2O		0,41 g/L	10 ml
Ferric citrate		88 mg/L	8 ml
L – Glutamine		0,6 g/L	10 ml
L – Leucine		0,1 g/L	10 ml
DL – Isoleucine		0,1 g/L	10 ml
DL – Valinie		0,1 g/L	10 ml
DL – Methionine		0,1 g/L	10 ml
L – Arginine Hcl		0,1 g/L	10 ml
L – Cysteine Hcl		0,1 g/L	1 ml
L – Histidine Hcl		0,1 g/L	10 ml
L – Tryptophan		0,1 g/L	10 ml
L – Phenylalanine		0,1 g/L	10 ml
Adenine		2,5 mg/L	0,5 ml
Biotin		0,5 mg/L	1 ml
Riboflavine		5 mg/L	20 ml
Thiamine Hcl		1 mg/L	1 ml
Pyridoxal HCL		1 mg/L	1 ml
Para – aminobenzoic acid		1 mg/L	1 ml
Calcium panthothenate		1 mg/L	1 ml
Nicotinamide		1 mg/L	1 ml
Thioctic acid (a-lipoic acid)		5 mg/L	100 µl
H_2O (deionised & autoclaved)			760 ml

Table 2. IMM composition.

5.1.3 *Listeria* inocula preparation

Bacteria pre-inocula was set up placing several well-isolated colonies from a pure culture into 10 ml of BHI broth for an overnight culture. The following day, bacteria from the overnight culture were inoculated 1:100 into 200 ml of BHI broth for a new incubation until reaching an OD_{600} of 1. Bacteria culture was centrifuged at 7,000 × *rpm* for 10 min at 4°C. Three washes in ice-cold PBS (Fluka-Sigma), were performed and the pellet from the last wash was re-suspended in 4 ml ice-cold 20% glycerol (Sigma) PBS. 100µl aliquots were dispensed into tubes and stored at -80°C until use. In order to determine the number of bacteria present in the frozen inocula stocks, three aliquots were serial diluted and plated in BHI and then the average CFU/ml was calculated.

5.1.4 Bacterial growth curve

Bacterial population size in broth culture can be estimated by measuring its turbidity using a spectrophotometer. The day before the experiment, a pre-inoculum was prepared as it has been previously specified in Section 5.1.3. This step is important to guarantee that all the bacteria are at the same stage by the time of the experiment. The day of the experiment, the overnight culture was centrifuge at 7,000 x rpm for 10 min and the pellet was washed twice with PBS to be finally resuspended in 10 ml of PBS. OD_{600} was measured and an appropriate volume of the bacteria in PBS was mixed with 1ml of selected media to reach an $OD_{600} \approx 0.02$. To set up the flat

bottom 96-well plate (Nunc Thermo Scientific), 200 µL of the bacteria in the appropriate media were added per well. Three technical replicates were made for each strain. Plates were at 37ºC shaking and measurements were taken every 30 min. A Fluorostar Omega plate Reader (BMG LABTECH) was used to measure the growth. Data were analyzed using MARS Data Analysis Software (BMG LABTECH).

5.1.5 Competent cells preparation

5.1.5.1 *L. monocytogenes* electrocompetent cells

The day before competent cells preparation, a pre-inoculum was prepared as it has been described in Section 5.1.3. The culture was diluted 1:100 in 0.5 M sucrose (Sigma) BHI and incubated under the same conditions until reaching an OD_{600} = 0.2. Then, 10 µg/ml penicillin G (Sigma) was added and the culture was incubated for further 2h under the same conditions. After that, bacteria were transferred to sterile ice-cold centrifuge tubes to spun them at 7,000 x *rpm* for 10 min at 4°C. Ice-cold transformation buffer was prepared by mixing 0.5 M sucrose, 1 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma) in Milli-Q water to wash the cells up to three times. The final pellet was re-suspended in 200 µl of ice-cold transformation buffer containing 10% glycerol. 50 µl aliquots were dispensed into sterile microfuge tubes previously chilled at -80°C. Competent cells were immediately stored at -80°C until use.

5.1.5.2 *E. coli* chemical competent cells

From an overnight pre-inoculum (preparation described in Section

5.1.3), an inoculum was set up diluting the overnight culture 1:100 in 100ml of LB broth media. Once inoculum reached an OD_{600} = 0.5, bacteria were passed to centrifuge tubes and incubated on ice for at least 10 min. After this time, tubes were centrifuged at 7,000 rpm during 10 min at 4ºC. The pellet was resuspended in 20 ml of Solution I and left again on ice for at least another 10 min. Bacteria were centrifuged under same conditions as before and the pellet was resuspended in 4ml Solution II. 150 µl aliquots were dispensed into sterile microfuge tubes and stored at -80°C.

Solution I: 10mM sodium acetate (AcNa, Sigma) pH=5.6-6, 50mM manganese (II) chloride (Cl_2Mn, Sigma) and 5mM sodium chloride (ClNa, Sigma). Solution II: 10mM AcNa pH=5.6-6, 5% glicerol, 70mM calcium chloride (Cl_2Ca, Sigma) and 5mM Cl_2Mn.

5.2 Molecular biology techniques

5.2.1 DNA techniques and sequence analysis

5.2.1.1 Polymerase chain reaction

The oligonucleotides used in this project (Table 3) were designed using *L. monocytogenes* EGDe genome as a reference (Glasser *et al.*, 2001) and purchased from Sigma Genosys. Genomic DNA was extracted from the bacteria using GenElute Bacterial Genomic DNA Kit (Sigma) following manufacturers instructions. Polymerase chain reactions (PCRs) were carried out using C100 thermal cycler (Bio-Rad).

Taq DNA polymerase (Biotools) was used for routine checking, following

general parameters: 5 min at 95°C of initial denaturation; 30 cycles of amplification involving 30s at 95°C of denaturation, 30s of oligonucleotide hybridization at the appropriate melting temperature and 2 min elongation at 72°C plus another 10 min also at 72°C of final elongation after the last cycle. A master mix volume of 25 µl per reaction was used for Taq polymerase PCRs mixing 100 ng of DNA template, 0.5 µl of 10 mM dNTPs (Biotools), 1 µl of each 10 µM oligonucleotides, 2.5 µl of Taq polymerase buffer and 1 µl 10x Taq polymerase.

PfuUltra II fusion HS DNA polymerase (Stratagene) was used when high-fidelity PCR was required. Thermal cycler parameters were set as follows: initial denaturation for 2 min at 95˚C; 30 cycles of amplification involving denaturation for 20s at 95˚C, oligonucleotide hybridisation for 20s at the appropriate temperature (Table 3) and elongation at 72˚C 30s; and final elongation for 5 min at 72˚C. 50 µl master mix was used according to the manufacturers conditions: 100 ng of DNA template, 0.25 µl of 10 mM dNTPs (Biotools), 0.5 of each 10 µM oligonucleotides, 5 µl of Taq polymerase buffer and 0.5 µl *PfuUltra II* polymerase.

PCR products were visualized on 1% agarose gels (Biotools) by running a gel electrophoresis.

Mutant/Complementation	Primer name	Sequence 5'-3'	Anealing temperature	Description
EGD,*glcU1*::pAUL-A EGDΔ*ptsH*,*glcU1*::pAUL-A	0184_INF	GGC**GGATCC**ATTGCTAGTTTTGTCTCT	55°C	Forward primer for the construction, Restriction site for Bam HI
	0184_INR	CCC**AAGCTT**TACCAGTAGCCCACATCACC	55°C	Reverse primer for the construction, Restriction site for HindIII
	0184_ext_F	GGGTATAGACCATAAGCAGA	55°C	Forward external primer to check insertion in the chromosome
	0184_ext_R	TATATCAAACCGGAAGCTGT	55°C	Reverse external primer to check insertion in the chromosome
EGDΔ*ptsH*(pPL2-Pδ*ptsH*)	ptsHC_F1_BamHI	CG**GGATCC**ATAGTCTTTTTGGCT	55°C	Forward primer for the construction, Restriction site for Bam HI
	ptsHC_R1_SpeI	G**ACTAGT**AGCTTTCGCAATGGC	55°C	Reverse primer for the construction, Restriction site for SpeI
	PL95	ACATAATCAGTCCAAAGTAGATGC	58°C	Forward ppl2 primer to check insertion in the chromosome
	NC16	GTCAAAACATACGCTCTTATC	58°C	Reverse ppl2 primer to check insertion in the chromosome
	ppl2 1R	CCGCCCTGCCACTCATCGCAG	58°C	Forward primer to check the construction
	ptsHC_FN	ACATGGTACTCTTTTTA	58°C	Reverse primer to check the construction
pAUL-A	paulaF	TGTAAAACGACGGCCAGT	58°C	Forward primer to check the insert inside pAUL-A vector
	paulaR	CAGGAAACAGCTATGAC	58°C	Reverse primer to check the insert inside pAUL-A vector

Table 3. Oligonucleotides used. The table details specific oligonucleotides sequences, restriction enzymes sites and anealling temperature conditions for each PCR reaction.

5.2.1.2 Sequence analysis

For cloning purposes, PCR products were purified from the agarose gel with QIAquick Gel Extraction Kit (Qiagen) following manufacturer conditions. Nanodrop(R) Spectrophotometer (ThermoScientific) was used to assess DNA purity and concentration. DNA samples with an A260/A280 absorbance ratio lower than 1.75 - 1.80 were discarded.

DNA samples were submitted to GenePool (University of Edinburgh) for Sanger sequencing. Sequences and chromatograms were analyzed using ApE plasmid Editor (Wayne Davis). The Alignment of DNA sequences was performed by using Basic Local Alignment Search Tool (Blast, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

5.2.1.3 Digestion and ligation

PCR and plasmid digestions were carried out using restriction enzymes. All enzymes were obtained from Promega (Biolabs) and they were used according to the manufacturer's instructions: 1 μg of purified PCR product/ plasmid was double digested within 50 μl reaction using 1μl of each restriction enzyme, 5μl of 10× bovine serum albumin (BSA) and 5 μl of appropriate reaction buffer. The digestion was incubated at 37°C during 2-24 h (depending on the enzyme) and subsequently PCR fragments were ligated with the plasmid using T4 ligase (Promega Biolabs) according to manufacturer's instructions: 100ng of DNA vector, 17ng of DNA insert, 1μ of Ligase 10× buffer T4 DNA, 1μl ligase and nuclease-free water to final

volume of 10µl were assembled in a sterile microcentrifuge tube. The reaction was incubated at 16 °C overnight and then incorporated by transformation into the appropriate bacterial strain.

5.2.2 Plasmid extraction and transformation

E. *coli* DH5α was used to clone and maintain plasmids. Vectors were introduced by heat shock: 10µl of the insert-vector complex were mixed with 75µl chemically competent *E. coli* (see section 5.1.5.2) and placed on ice for 30 min. After that time, mix was passed to a heat block at 42°C for 2 min, to come back to ice for farther 5 min. 900µl of BHI were added to the transformation mix to be then incubated for one hour at 37°C and subsequently plated in LB 250µg/ml erythromycin (LB Em 250) or LB 15 µg/ml chloramphenicol (LB Cm 15) plates as required. Plasmids were extracted from *E. coli* using QIAprep(®) Spin Miniprep Kit (Qiagen), according to manufacturer's instructions.

Plasmids were introduced inside *L. monocytogenes* by electroporation. *Listeria* electro-competent (see section 5.1.5.1) were defrosted on ice and mixed with 10 ng/µl of plasmid. The mix was transferred into an ice-cold 2 mm electroporation cuvette (Bio-Rad) and kept on ice for 5 min. Then the cuvette was placed in a GenePulserXcell electroporator (Biorad) with the following settings for electroporation: voltage = 2900 V, capacitance = 25 µF, resistance = 100 Ω. After transformation, 900 µl of BHI broth was added to the bacterial cells and the cuvette content was transferred into microfuge tube. Bacteria in BHI were incubated at 37°C for

1h and plated onto BHI agar plates containing the appropriate antibiotic (5μg/ml erythromycin or 7.5 μg/ml chloramphenicol). Transformants appeared within 24 – 48 h of incubation at 37°C.

5.2.3 Construction of insertion mutants in *Listeria*

L. monocytogenes EGDe insertion mutants were constructed using a modification of the homologous recombination technique previously described by Dominguez-Bernal *et al.* 2006.

Gene *glcU1* was amplified with 0184_INF and 0184_INR primers (Table 3) and cloned into pAUL-A plasmid (Chakraborty *et al.,* 1992) to generate pAUL-A*glcU1* that was introduced into *E. coli* . Transformants were restricted in LB Em 250 and checked by PCR (using PaulaF and PaulaR primers, figure 21). plasmid was extracted and electroporated in EGD-e. One colony carrying the construction was inoculated in 10 ml LB broth with 5 μg/ml , 20 μg/ml uridine (as an extra carbon source due to the incompetence of these strains to use glucose) and incubated at 30ºC overnight. The overnight culture was diluted in PBS 1:100 and 100 μl of the dilutions (10^{-3} to 10^{-6}) were plated out onto pre-warmed 42ºC BHI Em5 agar plates. Colonies that appeared after 48h incubating at 42°C were checked by PCR to select the strains EGD,*glcU1*::pAUL-A and EGD*ΔptsH,glcU1*::pAUL-A (Table 3).

5.2.4 Mutant complementation by site-directed mutagenesis

Complementation plasmid pPL2-P*δptsH* was constructed to restore the function of the Hpr protein from the PTS system. *ptsH* gene was

amplified by PCR with ptsHC_F1_BamHI and ptsHC_R1_SpeI primers (Table 3). PCR product was purified and cloned into the high-copy plasmid pCR Blunt vector (Invitrogen) in order to increase the insert concentration. After transforming into *E. coli*, the plasmid was extracted and digested with *SpeI* and *BamHI* to release the insert. Once purified, the digested insert was transferred to pPL2-Pδ vector. This plasmid was then introduced into *L. monocytogenes* EGD-e Δ*ptsH* by electroporation and the resulting colonies were checked by PCR.

pPL2-Pδ (Figure 22) is a modification of the original pPL2 plasmid (Lauer *et al.,* 2002) produced in our laboratory (de las Heras, unpublished data): the strong constitutive promotor Pδ was added to the site-specific phage integration vector pPL2 generating an integrative vector with a strong promotor to express a selected inserted gene. Thus, the expression of *ptsH* was driven by the Pδ promotor.

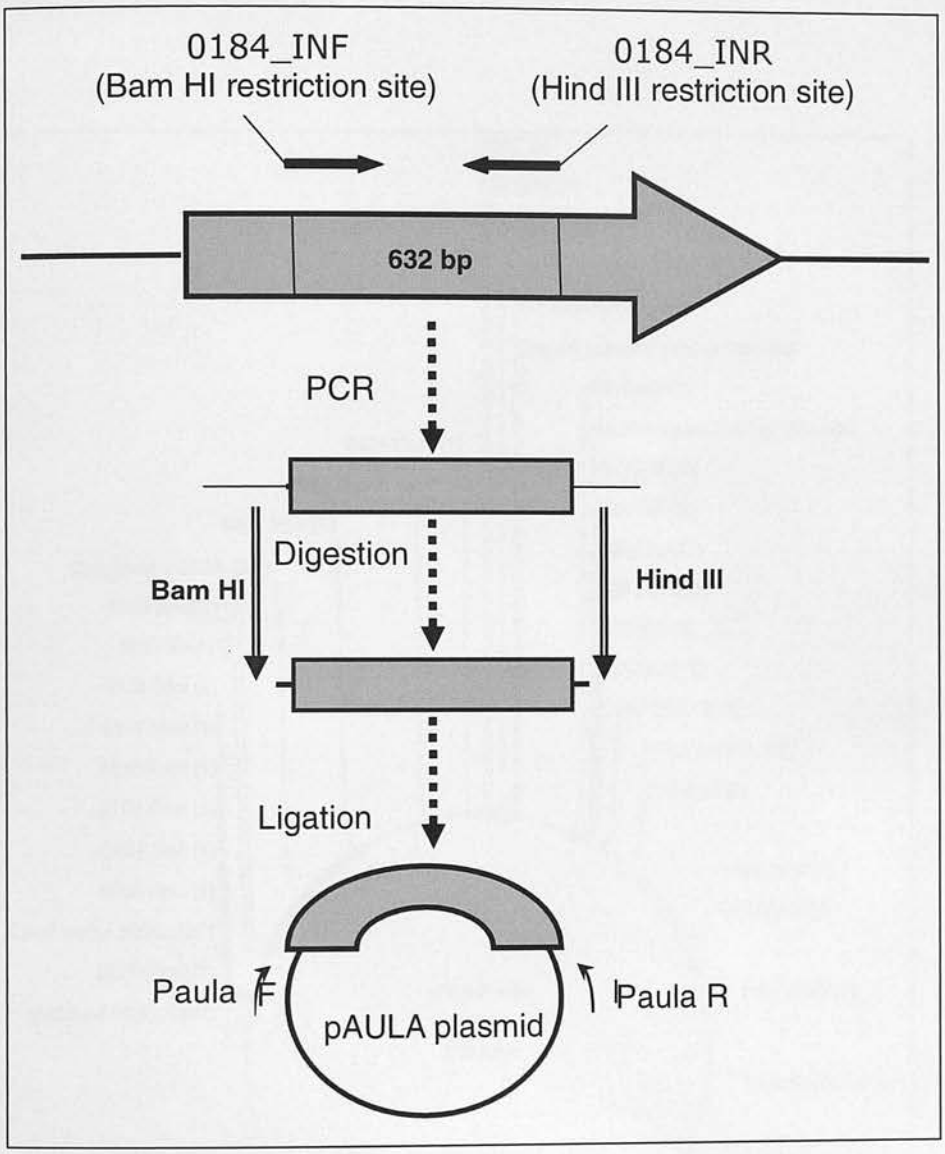

Figure 21. Schematic representation of the recombinogenic plasmid construction for an insertion mutant. The grey arrow represents the chromosomal gene to interrupt. Primers 0184_INF and 0184_INR (each of them with one restriction site at the 5' end) are represented with two small black arrows. Primers amplified a region of 632 bp in the middle of the gene (grey square represents), which was subsequently digested Bam HI/Hind III (small vertical pointed arrows) and inserted into pAUL-A, giving the recombinogenic plasmid. The presence of the insert inside the plasmid was checked by PCR using primers Paula F and Paula R.

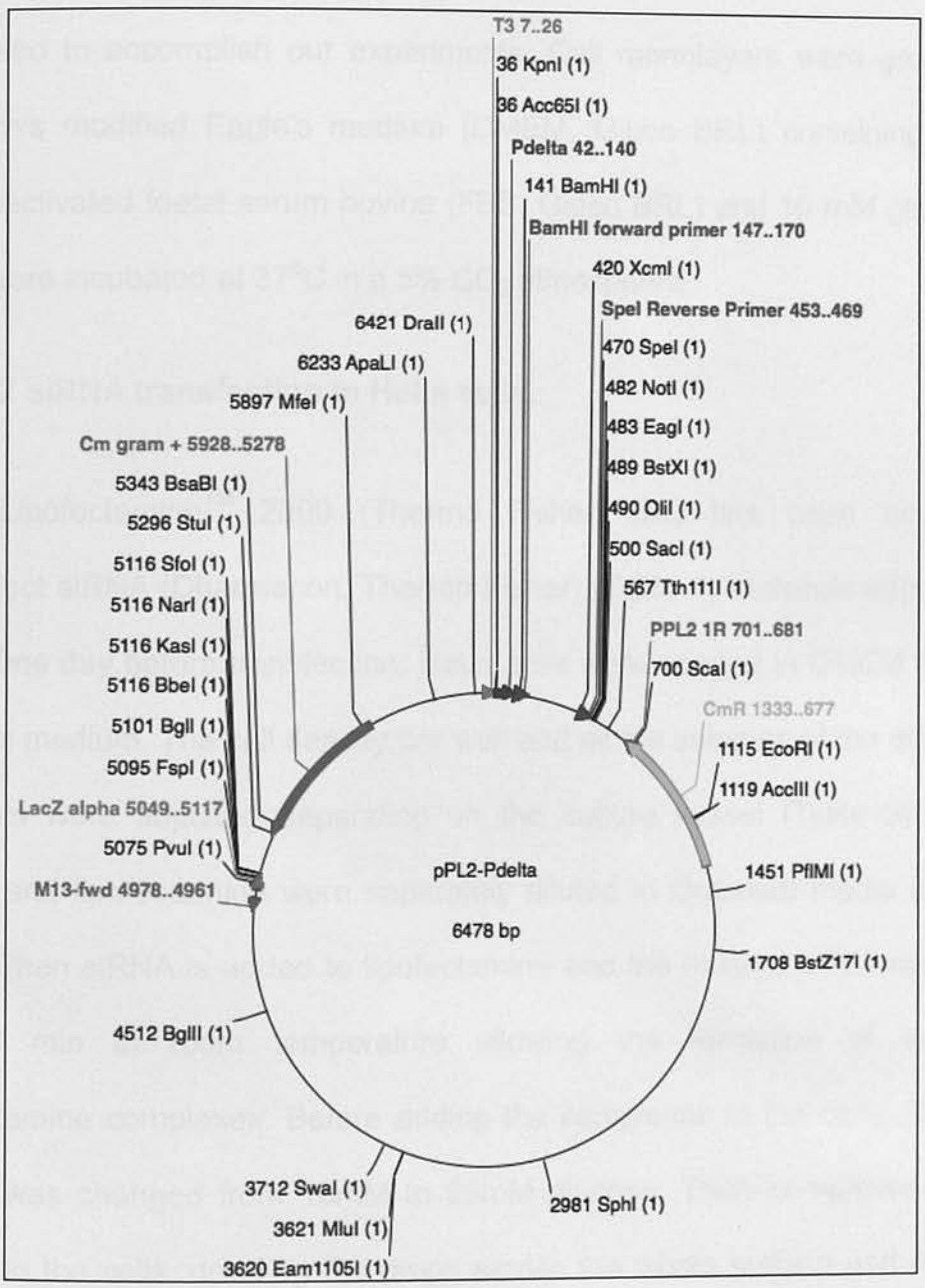

Figure 22. Schematic representation of pPL2-Pδ vector (de las Heras, unpublished data)

5.3 Cell-based techniques

5.3.1 Mammalian cell culture

The human epithelial cell line HeLa (CCL-2) from ATCC repository was used to accomplish our experiments. Cell monolayers were grown in Dulbeco's modified Eagle's medium (DMEM, Gibco BRL) containing 10% heat-inactivated foetal serum bovine (FBS, Gibco BRL) and 10 mM glucose. Cells were incubated at 37°C in a 5% CO_2 atmosphere.

5.3.2 siRNA transfection in HeLa cells

Lipofectamine™ 2000 (Thermo Fisher, UK) has been used to contrafect siRNA (Dharmacon, Thermo Fisher) (Table 4) molecule into HeLa cells. One day before transfection, HeLa cells were seeded in DMEM 10mM glucose medium. The cell density per well and all the volumes of the different reagents were adjusted depending on the culture vessel (Table 5). Both siRNA and lipofectamine were separately diluted in Optimem media (Gibco BRL). Then siRNA is added to lipofectamine and the mixture was incubated for 30 min at room temperature allowing the formation of siRNA-lipofectamine complexex. Before adding the complexes to the cells, DMEM media was changed from 10mM to 25mM glucose. Then complexes were added to the cells, drizzling the drops across the whole surface and mixing gently by rocking the plate back and forth. Cells were incubated at 37 °C, 5% CO_2 and after 4h growth, DMEM 25mM glucose was replaced with DMEM 5mM glucose and incubated at same conditions for 24h.

HEXOKINASE	SEQUENCE NUMBER	BRAND
HK1	D-006820-01	Thermo-Scientific
	D-006820-03	Thermo-Scientific
	D-006820-04	Thermo-Scientific
	D-006820-05	Thermo-Scientific
HK2	D-006735-02	Thermo-Scientific
	D-006735-03	Thermo-Scientific
	D-006735-04	Thermo-Scientific
	D-006735-05	Thermo-Scientific
HK3	D-006736-01	Thermo-Scientific
	D-006736-02	Thermo-Scientific
	D-006736-03	Thermo-Scientific
	D-006736-04	Thermo-Scientific
GCK (HK4)	D-010819-01	Thermo-Scientific
	D-010819-02	Thermo-Scientific
	D-010819-03	Thermo-Scientific
	D-010819-04	Thermo-Scientific

Table 4. Table containing siRNA used in this project.

Culture vessel	Cell no.	Relative surface area	plateing media (µL)	siRNA (20 µM)		Lipofectamine	
				oligo (µL)	media (µL)	oligo (µL)	media (µL)
96 well	7000	1	100	0.25	10	0.2	10
24 well	35000	5	500	1.25	50	1	50
12 well	70000	10	1000	2.5	100	2	100
6 well	100000	25	1500	6.25	250	5	250

Table 5. siRNA volumes and conditions for transfection

5.3.3 Preparation of HeLa cell extracts

Extracts from transfected HeLa cells (see section 5.3.3) were prepared 4h after transfection. Cells from 3 wells from a six-well plate made one extract. Media was removed from the cell plate, cells were washed twice with Dulbeco modified PBS (D-PBS) and placed on ice. Cell monolayers were scrapped in the presence of 80 µl/well of lysis buffer containing 50mM Tris hydrochloric (HCl, Sigma) ph7.4; 150 mM sodium choride (NaCl, Sigma); 1mM EDTA(Sigma); 1% tTriton-X-100 (Sigma); 0.5 sodium fluoride (NaF, sigma); 1mM sodium orthovanadate (Na_3VO_4, Sigma); 1 tablet of protein inhibitor cocktail EDTA free (Roche); 1mM tosyllysine chloromethyl ketone hydrochloride (TLCK, Roche); 1mM dithiothreitol (DTT, Roche). Extracts were collected in a microtube and placed on ice for 30 min vortexing from time to time. Then, extracts were centrifuge at 4°C, 10,000 rpm during 10 min. Supernatant was collected and frozen at -80 until analysis.

5.3.4 Intracellular proliferation assays

HeLa cells were cultured in a 24-well plate (Costar®-Corning) at a concentration of 3.5×10^4 cells/well, incubated at 37°C under 5% CO_2 in DMEM 10% FBS. Bacteria were added to cells using the appropriate MOI and the infected monolayers were centrifuged at 900 rpm for 3 min at room temperature to synchronize the infection. After centrifugation, infected cells were incubated for 20 min at 37°C in 5% CO_2 atmosphere. Cell monolayers were washed twice with D-PBS (Sigma) to remove non-adherent bacteria, and incubated during 1h in DMEM containing 25 µg/ml gentamicin (Sigma) to

kill live extracellular bacteria. Infection time 0 was determined 1h after the addition of gentamicin. At specific time points, two wells per strain were washed twice with D-PBS and cells were lysed by adding 100 µL 1% Triton X-100 (Sigma) for 3 min, and subsequently 400 µL of D-PBS. Appropriate dilutions were then performed for bacterial counting on BHI agar. IGC was used to normalised the intracellular bacterial growth data for the bacterial population at $t = 0$.

$$IGC \quad \frac{(IB_{t=n} - IB_{t=0})}{IB_{t=}}$$

$IB_{t=n}$ numbers of intracellular bacteria at time points $t = n$

$IB_{t=0}$ numbers of intracellular bacteria at time points $t = 0$

5.4 Biochemistry

5.4.1 Sugar-phosphates fermentation assay

The capacity of the bacteria to utilize HPs was measure in phenol red broth supplemented with glucose-1-phosphate (G1P) or G6P (Sigma) at a final concentration of 10mM. A stock of 100 ml phenol red broth was prepared by mixing 1.5 g of phenol red broth in 100ml of distilled water. To prepare the phenol red charcoal, 0.2 g of activated charcoal (Merk) were added 100ml of the phenol red broth. A single colony was cultured in 1 ml G1/6P-supplemented phenol red broth at 37°C during at least 24 h. Results reading was performed at 24h and 48h.

5.4.2 Measurement of lecithinase activity

The activity of the PrfA-dependent PlcB gene was semi-quantified in BHI agar plates supplemtned with lecithin solution with or without activated charcoal. The lecithin solution was prepared by mixing one egg yolk with 100 ml of filter sterilized 0.9% saline solution. 10ml of the lecithin solution were mixed with 100 ml of autoclaved BHI agar (prepared by mmixing 3.7 g of BHI (BD, Bacto™) and 1.6 g of Agar (OXOID) in 100 ml of distilled water). 13ml of media were pour per Petri dish. Lecithin charcoal plates were prepared on the same way with the addition of 0.5 g of charcoal to the BHI agar before autoclaving (BD, BBL™). Single colonies were streaked onto the plates and incubated at 37° C during 24 – 48 h. Lecithin activity was measured by the presence of a turbid halo around the positive colonies.

5.4.3 SDS-PAGE electrophoresis

Supernatant from cell extracts was mixed with 4× loading buffer (Sigma) and loaded in a NuPage precast gel (Life Technologies – Invitrogen). Samples were run in 1× MOPS SDS-PAGE running buffer (3-(N-morpholino) propanesulfonic acid for Sodium dodecyl sulfate polyacrylamide) gel electrophoresis (Novex- Life Technologies) in a Novex mini cell tank (Invitrogen, UK) and Power Pac 300 (Bio-Rad). Once samples were loaded, gel was initially run at constant amperage of 40 mV until the samples start to move into the gel properly. When samples got around 10 mm inside the gel, amperage was increased to 80mV until the gel front reached the bottom of the gel. Proteins were then transferred from the gel onto a polyvinylidene difluoride membrane (PVDF, GE Health Care) by electrophoresis.

5.4.4 Western blotting

A western blotting cassette (Bio-Rad) was placed into a shallow tray part filled with 1× transfer buffer containing: 10% 10× buffer stock, 20% methanol (Sigma,) and 70% distilled water. 10× stock buffer was prepared by mixing 144g of glycine (Sigma), 0.4% sodium dodecyl sulfate (SDS, Invitrogen) and 36g of TRIS base (tris(hydroxymethyl)aminomethane, Sigma). All membranes and filters were kept wet during the blotting stack construction to avoid the dehydration of gel and membrane that could disturb the transference. In order to build the blotting stack, a fibber pad support was placed at the bottom of the cassette. Then, two layers of Whatman paper (Whatman) cut to size were placed at the top of the pad followed by a

NuPage gel, the Polyvinylidene fluoride (PVDF) membrane (previously activated wetting it in methanol) and another two layers of Whatman paper. A circular length of pipette was rolled across the top of the Whatman paper in order to squeeze out any bubbles between the layers that could disrupt the transfer. Transfer buffer was added over the stack for each additional layer. Another fibre pad was added to the top of the stack and then the cassette was closed and fitted into the blotting tank set up according to manufacturers directions. The blot was transferred at a constant voltage of 30mV overnight. Then the membrane was removed from the cassette ready for the antibody detection.

5.4.5 Antibody detection

The membrane was blocked with 5% of skimmed milk diluted in Tris-Buffered Saline with Tween 20 (TBST, Sigma) during 1h at room temperature in a rolling shaker. Once the block was removed, the first antibody (Table 6) appropriately diluted in 5% skimmed milk TBST, was added. The membrane was incubated with the primary antibody for 1h under the same conditions mentioned above. After that, the membrane was washed three times with TBST buffer, with a period of 15 min per wash. Once the membrane is clean of primary antibody rests, appropriately diluted HRP-secondary antibody (Table 6) was added to be incubating for 1h. Finally, the membrane was again washed three times with TBST buffer and developed using ECL Plus Western Blotting Detection kit (GE Healthcare, Life Science) following manufacturers instructions.

Name	Host Species	Storage T	Company	Concentration
Hexokinase 1	Rabbit	-20C	Cell Signalling	1:1000
Hexokinase II	Rabbit	-20C	Cell Signalling	1:1000
Anti-HK-3 C-Terminal	Rabbit	-20C	Sigma	1:1000
Monoclonal Anti-GCK	Mouse	-20C	Sigma	1:2500
HRP Anti-Rabbit IgG	Donkey	4C	Amersham	1:5000

Table 6. Table containing the antibodies used in this project

5.4.6 Determination of intracellular concentration of glucose and glucose phosphate

HeLa cells from three 60mm Petri dishes made one extract. Cell monolayers were scrapped in presence of 100 μL of PBS 10% perchloric acid (Sigma) per extract and then centrifuged at 10,000 rpm at 4^0C for 15 min. The supernatant was collected to measure glucose and G6P after neutralizing the pH by adding 5 M potassium carbonate (K_2CO_3, Sigma) (Lang and Michal, 1974). The pellet was used to determine the total amount of protein in the extract using a quantification kit (Thermo Scientific) based on the pyrogallol red protein determination method (Anderson *et al.,* 1981). Basically, the pellet was resuspended in 150 μL of 30% potassium hydroxide (KOH, Sigma) and the mixture was boiled at 100^0C for 15 min. 15 μL of the protein dilution were mixed with 200 μL of the Microprotein reagent (Thermo Scientific) and the absorbance was measured at 600nm.

The total amount of glucose of each extract was determined by using a commercial kit (Thermo Scientific) based on the HK/G6P dehydrogenase (G6PDH) method (The American Association of Clinical Chemistry). HK catalyses the phophorylation of glucose by ATP producing G6P. G6P is at the same time oxidized to 6-phosphogluconate with the reduction of NAD+ to NADH by G6PDH. The amount of NADH formed is proportional to the concentration of glucose and can be measured by the increase in the absorbance at OD_{340}. G6P was determined by a spectrophotometric assay using G6PDH, which oxidises the sugar phosphate into 6-

phosphogluconolactone. This compound converts spontaneously into 6-phosphogluconate generating NADH that is proportional to the G6P and can be quantified as described previously in this section.

The final intracellular glucose concentration was estimated using following calculations:

$$\text{Intracellular glucose concentration} = \frac{Glc_{sb} - G6P_{sb}}{Cell_{vol}}$$

$$Cell_{vo} = \text{mg protein cell extract} \times \frac{0.64 \text{ mL cell volume}}{120\text{mg}}$$

Glc_{sb} : μ m glucose in cell extract supernatant

$G6P_{sb}$: μ m glucose in cell extract supernatant

$Cell_{vol}$: extracts cell volume

5.5 Microscopy techniques

5.5.1 Actin staining

The protocol to develop this assay was detailed in Deshayes *et al.* (2012), briefly: 5×10^4 HeLa cells/well were seeded in a 24 well-plates with a

coverslip in each well. Next day, cells were infected at a suitable MOI and sampled 4h after infection. Then, infected monolayers were washed three times in warm D-PBS to remove extracellular bacteria and 100 mg/ml gentamicin (Invitrogen) was added after 1h of infection to prevent extracellular bacterial growth. Coverslips were fixed with 3.7% (w/v) paraformaldehyde (Invitrogen) and prepared for microscopic examination by permeabilization with 0.2% (v/v) Triton X-100 (Sigma) in PBS for 15 min. A further incubation of 1 h with 1:1,000 AlexaFluor 568-conjugated phalloidin (Invitrogen) and 1:50,000 DAPI (Invitrogen) was performed to visualize F-actin and bacterial DNA/cell nuclei, respectively. After three washes in PBS and one wash in distilled water, coverslips were mounted using ProLong Anti-fade reagent (Invitrogen) and mounting medium (Invitrogen). Images were acquired using 63 x oil objective in a Leica DMI 6000B immunofluorescence microscope and images were analyzed using Leica Application suit advanced fluorescence software (LAS-AF, Leica Microsystems).

Quantification of vacuolar and cytosolic *Listeria* was performed by counting the proportion of bacteria associated/ non-associated with F-actin per image field.

5.6 Statictical analysis

For the comparisons of sample means in intracellular proliferation assays a Student's t-test was perform to analyse the data.

6. References

6.1 References

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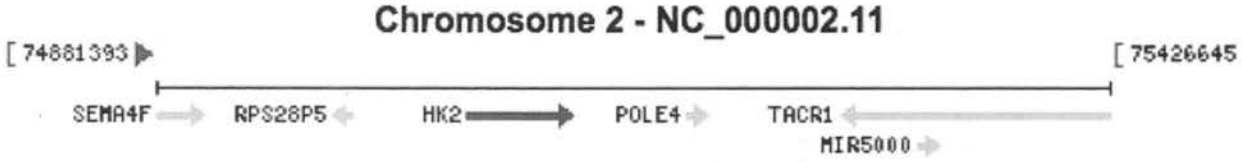
7. Appendix

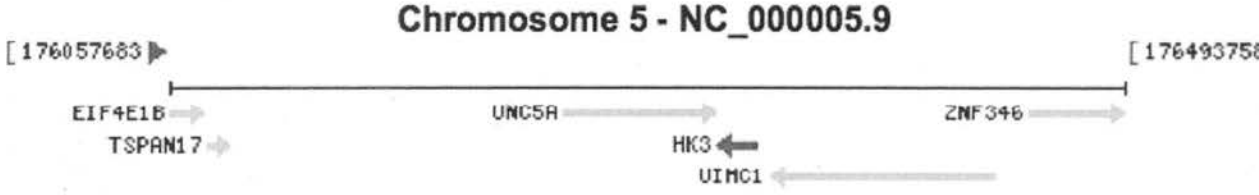
Appendix 1: Chromosomal site of HeLa cell Hexokinases

Hexokinase 1
Location: 10q22
Sequence: Chromosome: 10; NC_000010.10 (71029756..71161638)

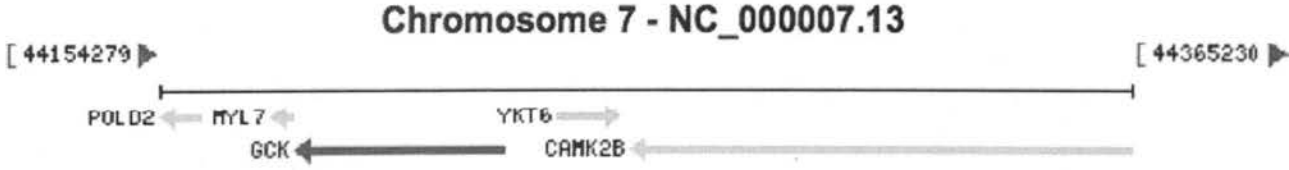
Hexokinase 2
Location: 2p13
Sequence: Chromosome: 2; NC_000002.11 (75059782..75120481)



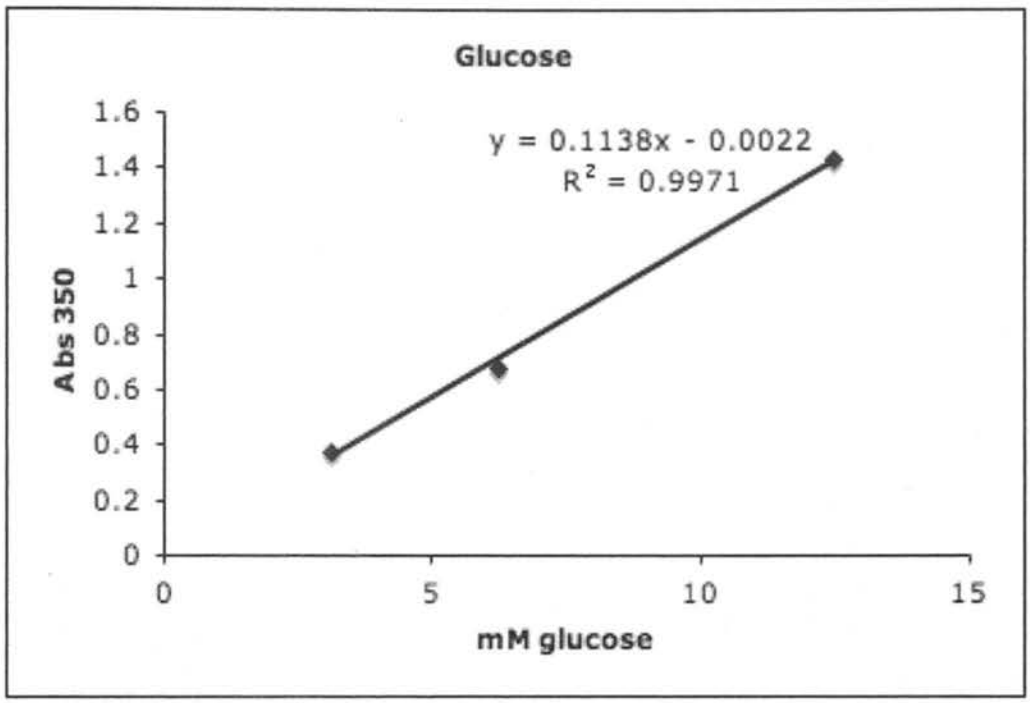
Hexokinase 3
Location : 5q35.2
Sequence : Chromosome: 5; NC_000005.9 (176307870..176326333, complement)

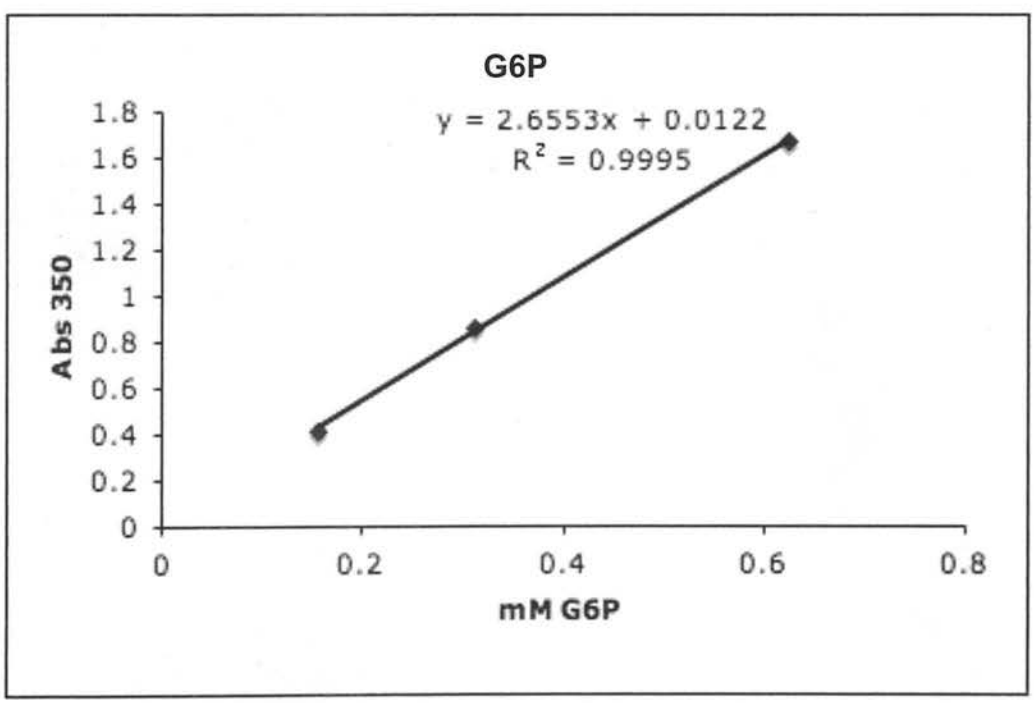


Glucokinase
Location : 7p15.3-p15.1
Sequence : Chromosome: 7; NC_000007.13 (44183870..44229022, complement)

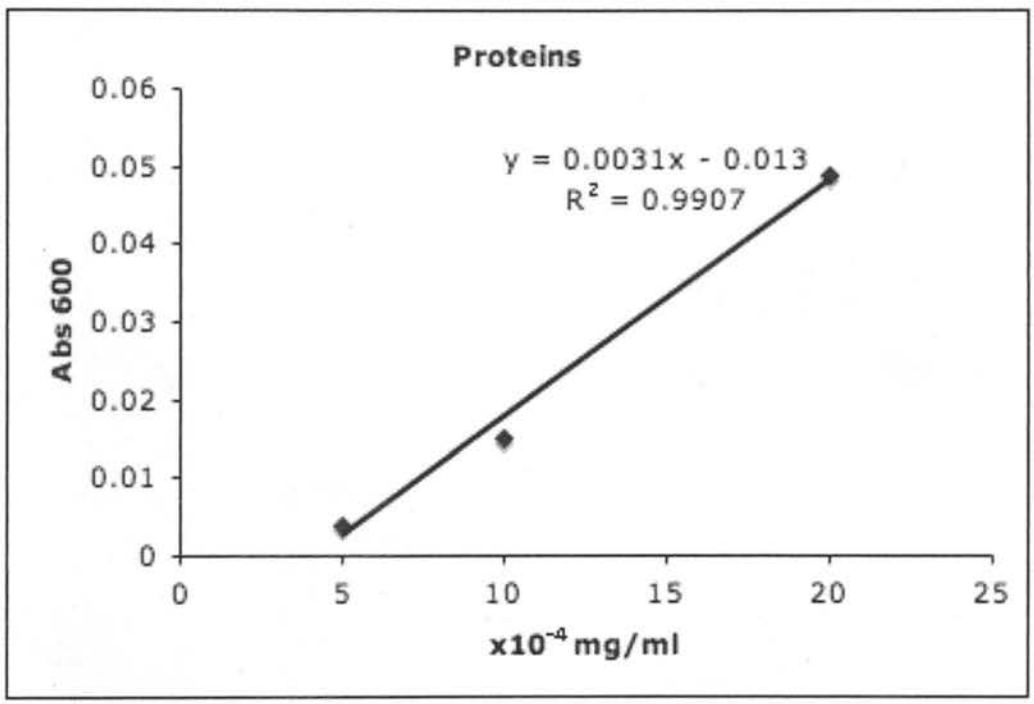


Appendix 2: Trendlines for the determination of the intracellular glucose and G6P concentration in HeLa cells





Appendix 3: Trendline for the determination of the total protein concentration in HeLa cells



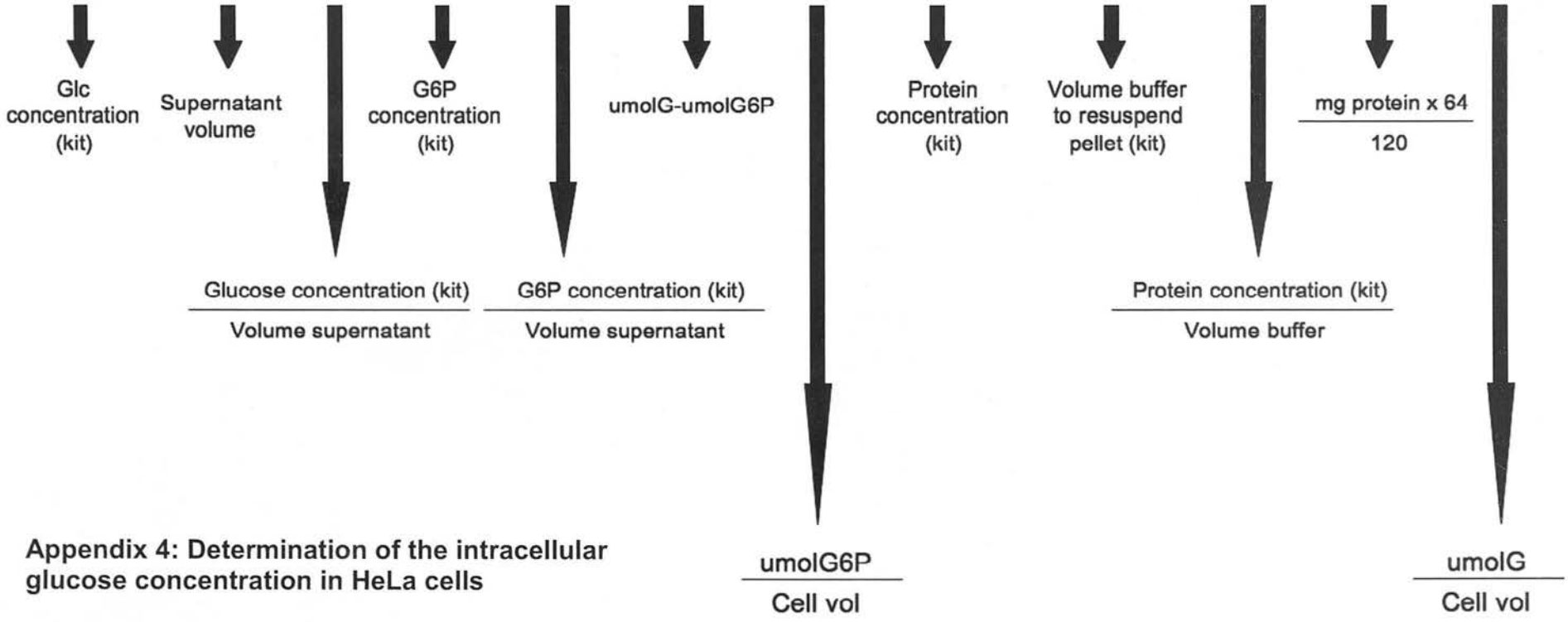
0.104	0.67	0.06968	0.000368	0.0002466	0.0694334	0.253	1.22	0.15	0.183	0.001	71.141
0.083	0.42	0.03486	0.000427	0.0001793	0.0346807	0.140	1.6	0.15	0.24	0.001	27.094
0.10033333	0.53333333	0.05422667	0.00050633	0.00026505	0.0539616	0.227	1.459666667	0.15	0.21895	0.001	46.211

siRNA control

Glc(mM)	Sb vol (ml)	umol Glucose	Glc 6P(mM)	umol Glu6P	Glu- Glu6P	mM Glc6P	prot pellet (mg/ml)	vol (mL)	mg prot total	cell vol (ml)	mM glu intra
0.104	0.65	0.0676	0.00357	0.0023205	0.0652795	0.871	3.332	0.15	0.4998	0.003	24.490
0.044	0.625	0.0275	0.0043	0.0026875	0.0248125	1.541	2.18	0.15	0.327	0.002	14.227
0.046	0.4	0.0184	0.008	0.0032	0.0152	2.500	1.6	0.15	0.24	0.001	11.875
0.06466667	0.55833333	0.03783333	0.00529	0.002736	0.0350973	1.443	2.370666667	0.15	0.3556	0.002	18.506

no siRNA

Glc(mM)	Sb vol (ml)	umol Glucose	Glc 6P(mM)	umol Glu6P	Glu- Glu6P	mM Glc6P	prot pellet (mg/ml)	vol (mL)	mg prot total	cell vol (ml)	mM glu intra
0.034	0.53	0.01802	0.0023	0.001219	0.016801	0.964	1.58	0.15	0.237	0.001	13.292
0.032	0.56	0.01792	0.0014	0.000784	0.017136	0.454	2.16	0.15	0.324	0.002	9.917
0.038	0.35	0.0133	0.001	0.00035	0.01295	0.259	1.69	0.15	0.2535	0.001	9.578
0.03466667	0.48	0.01664	0.00156667	0.000752	0.015888	0.519	1.81	0.15	0.2715	0.001	10.972



Appendix 4: Determination of the intracellular glucose concentration in HeLa cells